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(71) Applicant: **CHIRON CORPORATION**
4560 Horton Street
Emeryville California 94608(US)

(72) Inventor: **Dina, Dino**
1254 Washington Street
San Francisco California 94108(US)
Inventor: **Potter, Steven J.**
132 Ross Valley Drive
San Rafael California 94901(US)
Inventor: **van Nest, Gary A.**
4898 San Pablo Dam Road
El Sobrante California 94803(US)
Inventor: **Caput, Daniel**
3986 23rd Street
San Francisco California 94114(US)

(74) Representative: **Goldin, Douglas Michael et al**
J.A. KEMP & CO. 14, South Square Gray's Inn
London WC1R 5EU(GB)

(54) **Hepatitis a virus vaccines.**

(57) The complete genome of the hepatitis A virus - (HAV) is disclosed. With the entire genomic sequence available, epitopes are identified by analogy to known picornaviruses such as polio virus, synthesized chemically, and conjugated to carrier proteins to provide protective vaccines against HAV. Alternate methods of preparing vaccines include preparation of specified peptide sequences using recombinant techniques, preparation of deleted virions by modifying the genome to destroy the replicase and LTR regions, and preparation of particulate immunogenic materials containing an epitope from HAV. The foregoing alternatives for vaccine preparation are described, and proteins thus prepared used to raise antibodies against HAV.

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HEPATITIS A VIRUS VACCINES

Technical Field

This invention relates to the field of recombinant vaccines and viral diseases. More particularly, the invention relates to use of the hepatitis A virus genomic sequence to obtain oligomeric probes for diagnosis, to obtain vaccines useful in protection of subjects at risk with respect to hepatitis A, and to obtain monoclonal antibody populations useful in treatment of infected subjects and as diagnostics.

Background Art

Hepatitis is a serious debilitating disease prevalent throughout the world. It may occur in acute form and causes extensive fatigue, liver damage, and, not infrequently, is fatal. The disease is caused by a viral infection. The infective agents are endemic in underdeveloped areas of the globe, where the majority of adults carry antibodies directed against viral antigens. In the United States, Western Europe, and Japan, the incidence of hepatitis is also significant especially among those exposed to conditions which are conducive to transmission of viral infection. However, because the probability of exposure in general is lower, most adults in these areas do not carry antibodies directed against the antigens of the virus causing hepatitis, and are at high risk when travelling to areas where the virus is endemic.

Three very different and immunologically distinct forms of virus causing hepatitis are known: Hepatitis A, Hepatitis B, and a third known at present only as Non-A/Non-B. Hepatitis B virus - (HBV) has been widely studied and vaccines protective against infection by HBV have been developed. Another infective species, hepatitis A virus - (HAV) is a picornavirus belonging to the enterovirus genus. HAV was first characterized as the cause of acute illness by Feinstone, S. M., et al, Science - (1978) 182:1026-1028; Siegl, G., et al, J Virol - (1978) 26:40-47, 48-53. This form of the virus causes a clinically important disease, and more than 100,000 cases of HAV-induced hepatitis are reported annually in the United States. The incidence of the disease is similar elsewhere in the Western world.

As antibodies raised against hepatitis B are not cross-reactive against hepatitis A or protective against HAV infection, there is a need for the development of vaccines specifically directed against infection by HAV. While the virus has been characterized morphologically, and the genome partially sequenced, the development of a vaccine has been hindered by insufficient knowledge of the nucleotide sequence of the genome. As will be further explained below, vaccine design is dependent on identification of suitable epitopes either as primary peptide sequences, or resulting from production of 3-dimensional configurations, which engender the formation of neutralizing antibodies.

HAV has the typical icosahedral structure of a picornavirus, measuring 27-32 nm in diameter. The virions contain a single stranded genomic RNA molecule of approximately 7.5 kb, having positive strand polarity with a polyA stretch at the 3' end, and may be covalently linked to a protein at the 5' terminus. It has been assumed that the polypeptides encoded by the RNA are analogous to those of other picornaviruses, such as those responsible for polio and for foot and mouth disease (FMD). While variations occur, sufficient similarity has been shown between such viruses to warrant devising a systematic nomenclature of picornavirus proteins, as set forth in Rueckert, R. R., et al, Journal of Virol (1984) 50:957-959. This system of nomenclature, called the L434 convention, is based upon an idealized map of a polyprotein produced by the picornavirus genome, which can be accomodated into the pattern L-ABCD-ABC-ABCD. In this notation L is a leader protein, the first quartet represents a set of 4 capsid proteins encapsulating the viral particle, the middle triplet represents proteins of various functions, and the downstream quartet includes a proteinase capable of cleaving the polypeptide precursor of the capsid proteins into individual peptides. As shown by the invention herein, this pattern is consistent for HAV encoded polypeptides as well.

Some work relevant to vaccine preparation has been done. Monoclonal antibodies (Mabs) against HAV which are neutralizing in in vitro assays, but not protective against infection, were prepared by MacGregor, A., et al J Clin Microbiol (1983) 18:1237-1243, using spleens from mice injected with whole virus as fusion partners in preparing the Mab producing hybridomas. These Mabs appear cross-reacting, and apparently recognize antigenically similar determinants. In addition, Hughes, J. V. et al, J Virol (1984) 52:465-473 prepared Mabs which were capable of immunoprecipitating whole HAV, but not the disrupted or denatured proteins of the virus. These Mabs were obtained from hybridomas which had been formed using as fusion partners the spleens of mice which had been injected with a mixture of VP1, VP2, and VP3 HAV proteins. (Consistent with other picornaviruses, the

capsid proteins appear to comprise three or four separate proteins, VP1, VP2, VP3, and possibly VP4, of which VP1 is apparently the most exposed, and the major provider of antigenic determinants.) It has been consistently shown that the whole virus contains antigenic sites which are not present in individual capsid proteins. (Gerlich, W. H., et al, Med Microbiol Immunol (1983) 172:101-106; Hughes, J. V. et al (supra).)

Emini, E.A. et al, Abstracts; Modern Approaches to Vaccines (Sept 12-16, 1984, Cold Spring Harbour, NY) p72, have reported that poliovirus peptides corresponding to poliovirus capsid antigenic sites, when conjugated to BSA and injected into rabbits, rats or guinea pigs, were able to prime the subject to respond to sub-immunogenic adminstration of HAV by producing neutralizing antibodies.

Partial cloning and sequencing of the HAV genome has been reported by others (Ticehurst, J. R., et al, Proc Natl Acad Sci (USA) (1983) 80:5885-5889; Von der Helm, et al, J Virol Meth (1981) 3:37-44). These reports have yielded insufficient information to permit construction of polypeptides which can serve as vaccines. The present invention provides the entire genomic sequence of HAV, and thus permits manipulation of the potential epitopes to obtain effective vaccines against HAV.

Disclosure of the Invention

The invention provides a cDNA replica of the entire HAV genomic sequence. Portions of the genomic sequence are useful as probes to diagnose the presence of virus in clinical samples. The understanding of the genomic sequence also effectively makes available the entire viral polypeptide sequence and permits not only analysis, but production of portions thereof, if appropriate, in the correct configuration, so as to provide effective vaccines and permit the production of neutralizing antibodies. The availability of the entire HAV sequence thus permits the design and construction of polypeptides which may serve either as vaccines themselves, or as intermediates in the production of monoclonal antibody (Mab) preparations useful in passive immunotherapy against the disease, or as diagnostic reagents. Without the sequence of the entire genome at the disposal of the producer of the therapeutic or preventive compositions, successful production of optimally effective products would be impossible.

Accordingly, in one aspect, the invention relates to a nucleotide sequence substantially identical with that representing the entire genome of HAV as represented in Figure 1. The remaining contributions of the invention relate to utilizing this sequence or portions thereof as oligomeric probes, to produce peptides which can serve as vaccines, or as reagents to produce monoclonal antibodies useful in treatment of the disease.

Other aspects of the invention include expression systems which are capable of effecting the production of a desired protein encoded by sequences derived from the complete genome, to recombinant vectors containing such systems or portions thereof, to recombinant host cells transformed with such vectors, to proteins produced by the transformed cells, and to vaccines prepared from such proteins. In addition, the invention relates to specific peptide sequences representing epitopes encoded by the genome, and to such sequences covalently linked to carrier proteins. Such carrier proteins, in addition to more conventional carriers, include the 22 nm particle associated with hepatitis B infection, which carries polyalbumin receptor sites, and is 1000 fold more immunogenic than the unassembled subunit component. By inserting antigenic HAV determinants into the 22nm HBsAg particle, increased immunogenicity for these epitopes is obtained. The invention also relates to deleted virions capable of eliciting neutralizing antibodies in subject hosts, and to monoclonal antibodies useful in passive immunotherapy.

The invention also relates to the methods of preparing these desired polypeptide vaccines and immunoglobulins.

Brief Description of the Drawings

Figure 1 shows the complete nucleotide sequence of the HAV genome. The cDNA contains the identical sequence, except, of course, that T will be substituted for U. The deduced amino acid sequence of the viral L434 polypeptide is also shown in Figure 1.

Figure 2 shows a restriction map and the regions of overlap of the four cDNA clones which comprise the entire 7.5 kb HAV genome, pHAV47, pHAV8, pHAV1, and pHAV16.

Figure 3 shows the construction of expression vectors for sequences encoding fusion mRNAs encoding portions of superoxide dismutase (SOD) and HAV capsid protein.

Figure 4 shows a computer generated profile based on hydrophilicity and structural char-

acteristics of the peptide chain as a function of position for regions of the polio virus polypeptide and the HAV polypeptide.

Figure 5 shows the construction of a mammalian cell expression vector encoding deleted HAV virions.

Figure 6 shows the construction of pDC-104 for production of hybrid HAV/HBsAg in yeast.

Figure 7 shows the construction of pWG-1 for production of hybrid HAV/HBsAg in yeast.

Modes of Carrying Out the Invention

A. Definitions

As used herein, a nucleotide sequence "substantially identical" to the exemplified HAV genome refers to a sequence which retains the essential properties of the exemplified polynucleotide. A specific, but non-limiting example of such substantial equivalence would be represented by a sequence which encodes the identical or substantially identical amino acid sequence, but, because of codon degeneracy, utilizes different specific codons. Nucleotide changes are, indeed, often desirable to create or delete restriction sites, provide processing sites, or to alter the amino acid sequence in ways which do not adversely affect functionality. "Nucleotide sequence" refers both to a ribonucleotide and a deoxyribonucleotide sequence and includes the positive sense strand, as shown, and the negative sense strand as well.

A DNA sequence "derived from" the nucleotide sequence which comprises the genome of HAV refers to a DNA sequence which is comprised of a region of the genomic nucleotide sequence, or a combination of regions of the sequence. These regions are, of course, not necessarily physically derived from the nucleotide sequence of the gene, but refer to polynucleotides generated in whatever manner which have the same or "substantially identical" sequence of bases as that in the region(s) from which the polynucleotide is derived. For example, typical DNA sequences "derived from" the HAV genome include fragments encoding specific epitopes, fragments encoding portions of the viral polypeptide, sequences encoding the capsid proteins, sequences encoding deleted virions, and sequences encoding the viral proteinase. Similarly, a peptide "derived from" the HAV polypeptide refers to an amino acid sequence substantially identical to that of this polypeptide or a portion thereof, having the same biological properties as that portion. The manner of synthesis of such "derived" peptide is not material--it may be chemical synthesis or recombinant means, for example.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of the nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

"Control sequence" refers to DNA sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending on the host organism; in procaryotes, generally such control sequences include promoter and ribosome binding site; in eucaryotes, generally, such control sequences include promoter, terminators, and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

B. General Description

At the core of the present invention is the provision of a nucleotide sequence containing the entire genome of hepatitis A virus. The availability of this complete polynucleotide, first, permits the construction of oligomers of about 20 bp or more useful in disease diagnosis. Such probes may be used to detect the presence of the viral genome in,

for example, fecal filtrates of subjects suspected of harboring the virus. Knowledge of the gene sequence also enables the design and production of vaccines effective against HAV and production of neutralizing Mabs. Sequencing information available from the genome allows the amino acid sequence of the polypeptide to be deduced and compared to that of other known picornaviruses for which locations of favorable epitopes is known. Further, once the desired sequences are chosen, fragments of the genome can be obtained and expressed independently, thus providing desired polypeptides using recombinant techniques. Both procaryotic and eucaryotic hosts are useful for such expression. Short polypeptide fragments may be chemically synthesized and linked to carrier proteins for use as vaccines. Recombinantly expressed polypeptides may be provided under conditions offering a favorable environment for processing into, for example, deleted virions, thus permitting three dimensional reconstruction of epitopic sites without danger of creating an infective virus. Mammalian and yeast cells provide particularly suitable environments for such expression. In addition, the epitopes may be produced linked to a particle forming protein. The proteins thus produced may, themselves be used as vaccines, or may be used to induce immunocompetent B cells in hosts, which B cells can then be used to produce hybridomas that secrete antibodies useful in passive immunotherapy.

B.1. Preparation of the HAV Gene Sequence

The genomic sequence of HAV was prepared using stool samples of infected patients as the source for the virus. The isolated viral RNA was fractionated, and those fractions containing RNA of sufficient length to contain the entire genome were pooled, ethanol precipitated, and used to obtain a cDNA library. Colonies from the library were hybridized to oligomeric probes complementary to the 3'-proximal portions of the RNA. Several colonies hybridizing to these sequences were used to obtain additional portions of the RNA message from the cDNA library using "walking" techniques. Four cDNA clones were obtained from overlapping portions of the gene, and subjected to restriction mapping and sequencing. The entire genomic sequence was deduced from these four cDNA inserts. The amino acid sequence deduced from the viral genome is similar to that characteristic of picornaviruses, and the 5' and 3' non-coding regions probably involved in viral replication were identified.

B.2. Preparation of Viral Polypeptide Fragments in E. coli

The availability of the entire genomic sequence permits construction of expression vectors encoding presumptively antigenically active regions of the capsid proteins. Fragments encoding the desired proteins are obtained from the cDNA clones using conventional restriction digestion and ligated into vectors containing portions of fusion sequences such as β-glactosidase or SOD, preferably SOD. Any desired portion of the HAV genome could be expressed as a fusion protein or co-expressed with another readily produced protein in E. coli using this approach. However, for the purpose of generating peptides capable of raising antibodies against HAV, fragments representing portions of the genome encoding the capsid protein regions VP1, VP2 and VP3 were used. In a particularly preferred mode, human SOD encoding sequences are fused to those derived from HAV as described in more detail hereinbelow. Alternatively, since a three-dimensional antigenic site may result from suitable arrangement of individual peptide chains, bacterial expression vectors including the coding sequence for the viral proteinase, which would be encoded in the downstream quartet, may be included on the same, or cotransformed expression vector as that constructed for the precursor - (PI) of the capsid proteins. Simultaneous expression of the capsid encoding and proteinase encoding sequences may also result in preparations which produce the various capsid proteins as individual peptides. (See, e.g., Klump, W., et al, Proc Natl Acad Sci (USA) (1984) 81:3351-3355; Hanecak, R., et al, Cell (1984) 37:1063-1073.) These may then assemble to the appropriate three-dimensional structure.

B.3. Preparation of Antigenic Polypeptides and Conjugation with Carrier

The availability of the entire genomic sequence also makes possible computer analysis of the structural characteristics of the deduced protein for comparison with the known sequence of polio virus. As the antigenic regions of polio virus are known, such comparisons make possible the designation of short peptide regions representing epitopes. These can then be synthesized using chemical methods, and provided with, for example, cysteine residues at the C terminus which provide means for linking the peptides to neutral carrier proteins. A number of techniques for obtaining such linkage are known in the art, including the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-

thio)-propionate (SPDP) and succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate - (SMCC) obtained from Pierce Company, Rockford, Illinois. These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the ϵ-amino on a lysine, or other free amino group in the other. A variety of such disulfide/amide-forming agents are known. See, for example, Immun. Rev. - (1982) 62:185. Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid, and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. The foregoing list is not meant to exhaustive, and modifications of the named compounds can clearly be used.

Any carrier may be used, which does not itself induce the production of antibodies harmful to the host, such as the various serum albumins, tetanus toxoids, or keyhole limpet hemocyanin (KLH).

The conjugates, when injected into suitable subjects, will result in the production of antisera which contain immunoglobins specifically reactive against not only the conjugates, but also against fusion proteins carrying the analogous portions of the sequence, and against appropriate determinants within whole HAV.

B.4. Preparation of Non-Infectious (Deleted) Virions in Procaryotes or Eucaryotes

Virions which mimic the three-dimensional epitopic configuration of intact HAV may also be prepared. It is generally surmised that antigenic sites may result from three-dimensional folding of immunogenic proteins, and, specifically with respect to HAV, it has been reported that antibodies formed in response to injection with HAV capsid proteins are not reactive against the denatured viral peptides (Gerlich et al, Med Microbiol Immunol (1983) 172:101-106).

The deleted virions are prepared by providing DNA sequences which lack the 5' and 3' non-coding regions of HAV putatively responsible for replicative functions. The sequences also lack a portion of the 3' coding region presumed, by homology with other picornaviruses, to encode the replicase origin. These fragments are preferably cloned into plasmid expression vectors appropriate for yeast or mammalian cells capable of the desired post-transcription and post-translation processing; however, procaryotes can also be used. Deleted virions produced in hosts transformed with these vectors are immunoreactive against human convalescent HAV antisera, and can be used for immunization.

B.5. Preparation of Hybrid Particle Immunogens Containing HAV Epitopes

The immunogenicity of the epitopes of HAV may also be enhanced by preparing them in mammalian or yeast systems fused with particle-forming proteins such as that associated with hepatitis B surface antigen. Constructs wherein the HAV epitope is linked directly to the particle-forming protein coding sequences produce hybrids which are immunogenic with respect to the HAV epitope. In addition, all the vectors prepared include epitopes specific to hepatitis B virus (HBV), having various degrees of immunogenicity, such as, for example, the pre-S peptide. Thus, particles constructed from particle-forming protein which include HAV sequences are immunogenic with respect to both HAV and HBV.

Hepatitis surface antigen (HBsAg) has been shown to be formed and assembled in S. cerevisiae (Valenzuela et al, Nature (1982) 298:344-350), as well as in, for example, mammalian cells (Valenzuela, P., et al, Hepatitis B (1984), Millman, I., et al, ed, Plenum Press, pp. 225-236). The formation of such particles has been shown to enhance the immunogenicity of the monomer subunit. The constructs may also include the immunodominant epitope of HBsAg, comprising the 55 amino acids of the presurface (pre-S) region. - (Neurath et al, Science (1984) 224:392-394.) Constructs of the pre-S-HBsAg particle expressible in yeast are disclosed in U.S. Serial No. 621,756, filed 18 June 1984: hybrids including heterologous viral sequences for yeast expression are disclosed in U.S. Serial No. 650,323, filed 13 September 1984. Both applications are assigned to the herein assignee and incorporated by reference. These constructs may also be expressed in mammalian cells such as Chinese hamster ovary cells using an SV40-dihydrofolate reductase vector (Michelle et al, Int Symp on Viral Hepatitis (1984)).

In addition, portions of the particle-forming protein coding sequences per se may be replaced with codons for an HAV epitope. In this replacement, regions which are not required to mediate the aggregation of units to form immunogenic particles in yeast or mammals can be deleted, thus eliminating additional hepatitis B antigenic sites from competition with the HAV epitope.

B.6. Preparation of Vaccines

Preparation of vaccines which contain peptide sequences as active ingredients is also well understood in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified or the protein encapsulated in liposomes. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkaline glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipitents as, for example, pharmaceutical grades of manitol, lactose, starch magnesium stearate, sodium saccharine cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The protein may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practititioner and are peculiar to each subject.

B.7. Preparation of Mabs Against HAV Epitopes

The immunogenic proteins prepared as described above are used to immunize mammals. Lymphocytes from these animals may be used to prepare hybridomas capable of secreting monoclonal antibodies directed against these epitopes and cross-reactive against the infective virus. The resulting monoclonal antibodies are particularly useful in diagnosis, and those which are neutralizing are useful in passive immunotherapy.

C. General Methods

The general techniques used in extracting RNA from the virus, preparing and probing a cDNA library, sequencing clones, constructing expression vectors, transforming cells, and the like are known in the art and laboratory manuals are available describing these techniques. However, as a general guide, the following sets forth some sources currently available for such procedures, and for materials useful in carrying them out.

C.1. Hosts and Expression Control Sequences

Both procaryotic and eucaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences are used compatible with the designated host. Among procaryotic hosts, E. coli is most frequently used, mostly for convenience. Expression control sequences for procaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with procaryotic hosts are commonly derived from, for example, pBR322 a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance. The foregoing operons may be used as markers to obtain successful transformants by selection. Commonly used procaryotic control sequences include the β lactamase (penicillinase) and lactose promoter systems (Chang, et al, Nature (1977) 198:1056, the tryptophan (trp) promoter system (Goeddel, et al, Nucleic Acids Res (1980) 8:4057) and the λ derived P_L promoter and N gene ribosome binding site (Schimatake, et al, Nature (1981) 292:128) and

the hybrid tac promoter (De Boer, et al, Proc Natl Acad Sci (USA) (1983) 80:21-25) derived from sequences of the trp and the lac UV5 promoters. The foregoing systems are particularly compatible with E. coli; if desired other procaryotic hosts such as strains of Bacillus or Pseudomonas may be used, with corresponding control sequences.

Eucaryotic hosts include yeast and mammalian cell culture. Saccharomyces cerevisiae, or Baker's yeast and Saccharomyces carlsbergensis are the most commonly used yeast hosts, again because of convenience. Yeast compatible vectors carry markers which permit selection of successful transformants by conferring prototrophy to auxotrophic mutants or by conferring antibiotic resistance or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2 micron origin of replication (Broach, J., et al, Meth Enz - (1983) 101:307) the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences which will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors include promoters for the synthesis for glycolytic enzymes - (Hess, et. al, J Adv Enzyme Reg (1968) 7:149, Holland, et al, Biochemistry (1978) 17 :4900), and the promoter for 3 phosphoglycerate kinase - (Hitzeman, et al, J Biol Chem (1980) 255:2073). For yeast expression, terminators may also be included, such as those derived from the enolase gene - (Holland, M. J., J Biol Chem (1981) 256 :1385). Particularly useful control systems include those specifically described herein, which comprise the glyceraldehyde-3 · phosphate dehydrogenase (GAPDH) promotor or alcohol dehydrogenase - (ADH) regulatable promotor, terminators also derived from GAPDH, and, if secretion is desired, leader sequence from yeast alpha factor. These systems are described in detail in U.S. Serial Nos. 468,589 and 522,909, filed 22 February 1983 and 12 August 1983, respectively, both assigned to the herein assignee, and incorporated herein by reference.

Mammalian cell lines available as hosts for expression include many immortalized cell lines available from the American Type Culture Collection, including HeLa cells, Chinese hamster ovary - (CHO) cells, baby hamster kidney (BHK) cells, and a number of other cell lines. Suitable promoters for mammalian cells prominently include viral promoters such as that from Simian virus 40 (SV40) - (Fiers, et al, Nature (1978) 273:113 or other viral promoters such as the Rous sarcoma virus (RSV) adenovirus, and bovine papiloma virus (BPV). Mammalian cells may also require terminator sequences. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which insure integration of the appropriate sequences into the host genome.

C.2. Transformations

The transformation procedure used depends on the host to be transformed. Bacterial transformation generally employs treatment with calcium or rubidium chloride (Cohen, S. N., Proc Natl Acad Sci (USA) (1972) 69:2110, Maniatis, et al, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, page 254). Yeast transformations may be carried out using the method of Hinnen, et al, Proc Natl Acad Sci (1978) 75:1929-1933. Mammalian transformations are conducted using the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, or the various modifications thereof.

C.3. Vector Construction

Vector construction employs techniques which are by now quite well understood. Site-specific DNA cleavage is performed by treating with suitable restriction enzyme under conditions which generally are specified by the manufacturer of these commercially available enzymes (see, e.g., The New England Biolabs Product Catalog). In general, about 1 µg of plasmid or DNA sequence is cleaved by 1 unit enzyme in about 20 µl buffer solution for an incubation time of about 1-2 hr at about 37°C. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by reprecipitation with ethanol. The cleaved fragments may be separated using polyacrylimide or agarose gel electrophoresis techniques, according to the general procedures found in Methods in Enzymology - (1980) 65:499-560.

Sticky ended cleavage fragments may be blunt ended using E. coli DNA polymerase I (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) using incubation conditions appropriate to the polymerase. The polymerase digests protruding 3' single strands, but fills in 5' protruding ends, according to the dNTPs present in the mixture. Treatment with S1 nuclease may also be used, as this results in hydrolysis of any single stranded DNA portion.

Ligations are carried out using standard buffer and temperature conditions using T4 DNA ligase, and ATP; sticky end ligations require less ATP and less ligase than blunt end ligations. When vector fragments are used as part of a ligation mixture,

the vector fragment is often treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and thus prevent religation of the vector; alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent religation.

Ligation mixtures are transformed into suitable cloning hosts, such as E. coli , and successful transformants selected by, for example, antibiotic resistance, and screened for the correct construction.

C.4. Construction of Desired DNA Sequences

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer as described by Warner, B.D., et al, DNA (1984) 3:401-411. If desired, these synthetic strands may be kinased for labeling with ^{32}P by using an excess of polynucleotide kinase in the presence of labeled ATP, under standard kinasing conditons.

DNA sequences including those isolated from genomic or cDNA libraries may be modified by site directed mutagenesis, as described by Zoller, M, et al, Nucleic Acids Res (1982) 10:6487-6499. Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. The resulting double stranded DNA is transformed into a phage supporting host bacterium, and cultures of the transformed bacteria, which will contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically 50% of the new plaques will contain phage having as a single strand the mutated form: 50% will have the original sequence. Replicates of the plaques are hybridized to kinased synthetic probe at temperatures and conditions which permit hybridization with the correct strand, but not with the unmodified sequence. The thus identified, desired, modified sequences are then recovered and cloned to serve as sources for the desired DNA.

C.5. Hybridization with Probe

DNA libraries are probed using the procedure of Grunstein and Hogness (Proc Natl Acad Sci (USA) (1975)73:3961). Briefly, in this procedure, the DNA to be probed is immobilized on nitrocellulose filters, denatured, and prehybridized with a buffer containing 0-50% formamide, 0.6 M NaCl, 60 mM sodium citrate, 0.02% (wt/v) each of bovine serum albumin, polyvinyl pyrollidine, and Ficoll, 50 mM sodium phosphate (pH 6.5), 1% glycerine, and 100 µg/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps depends on the stringency desired. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those derived from cDNA or genomic sequences generally employ higher temperatures, e.g. about 40-42° and a high percentage, e.g. 50% formamide. Following prehybridization this same buffer, now containing the ^{32}P kinased oligonucleotide probe, is added to obtain hybridization. Radioautography of the treated filters shows the location of the hybridized probe, and the corresponding locations on replica filters which have not been probed can then be used as the source of the desired DNA.

C.6. Verification of Construction and Sequencing

For routine vector constructions, ligation mixtures are transformed into E. coli strain HB101 or other suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159, usually following chloramphenicol amplification (Clewell, D. B., J Bacteriol (1972) 110:667). The isolated DNA is isolated and analyzed by restriction analysis, or sequenced by the dideoxy method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463, as further described by Messing, et al, Nucleic Acids Res (1981) 9:309, or by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

D. Examples

The following examples are intended to illustrate but not limit the invention. The procedures set forth, for example, in ¶D.1 may, if desired, be repeated but need not be, as techniques are available for construction of the desired nucleotide sequences based on the information provided by the invention. Expression is exemplified in E. coli and in yeast, however other systems are available as set forth more fully in ¶C.1. Additional epitopes derived from the genomic structure may also be produced, and used to generate antibodies as set forth below.

D.1. Preparation of HAV cDNA

HAV virus was isolated from the stools of a hepatitis A patient collected during an epidemic outbreak in Los Angeles, California. Other isolates, available in the art could also have been used. See, for example, Daemer, R. J., et al, Infect Immunity - (1981) 32:388-393; Frosner, G. G., et al, Infection, - (1979) 7:303-350. Techniques for such isolation, and propagation are well known in the art.

The isolate was inoculated onto PCL/PRF/5 - (Alexander hepatoma cells) followed by culture at 34°C. Detectable virus production was observed 3-4 weeks later, and the original infected cell cultures have been continuously passaged in DME/15% FCS for the past two years with no loss of virus titers. (Virus titers were measured by immunofluorescence and/or competitive radio immunoassay (HAVAB, Abbott).)

Viral RNA was extracted from the virions by conventional procedures, for example, that disclosed by Ticehurst, J. E., et al, Proc Acad Sci - (USA) (1983) 80:5885-5889, including protease treatment and phenyl/chloroform extraction, followed by ethanol precipitation. Low molecular weight contaminants were fractionated from genome length RNA by 15-30% sucrose gradients run for 5 hr at 40,000 x g in a SW 41 Spinco rotor. Gradients were collected onto 22-24 fractions, each fraction precipitated, and run on a formaldehyde-agarose gel (Lehrach, H, et al,J. Biochem (1977) 16:474c. Each gel was transferred to a nitrocellulose membrane and hybridized to labeled specific probe as described by Thomas, Proc Natl Acad Sci (USA) (1980) 77:5201.

Prehybridization and hybridization were essentially as described above. Probes were kinased at the 5' terminus with T4 kinase according to the method of Lillehaug, et al, Biochemistry (1976) 15:1858 followed by purification on a Sep-pak - (Millipore) filter. Two oligonucleotide probes, designated HAV 1 and 2 were constructed based on sequencing information obtained by Ticehurst, et al, (supra). The probes had the following sequences:

HAV1: 3'-ACAAATAAAGAAAATAGTCATTTA-5'

HAV2: 3'-ATGTCTGAATTTAGAATACTAACCACC-5'

These probes were thus validated by hybridization to the gels run on HAV RNA from partially purified virions.

RNA from the sucrose fractions which contain full length HAV RNA were identified by the above-described hybridization blotting techniques. Samples were pooled, ethanol precipitated and used to form a cDNA library. The cloning procedure used is a modification of that previously described by Wood and Lee, Nucleic Acid Res (1976) 3 :1961; Lain et al, Cell (1979) 16:851. The two modifications introduced with respect to the published procedures are as follows: (a) a short double-stranded synthetic primer dT was annealed with the poly A+ RNA to synthesize the cDNA; (b) after dC tailing, the cDNA:RNA hybrid molecules were then annealed with PstI-cleaved dG-tailed pBR322. Before the transformation, the pBR322:mRNA:cDNA molecules were repaired with concentrated extract of activated Xenopus eggs which was prepared as indicated below. The ligation mixtures were than transformed into E. coli K12 MC1061 to Amp^R.

Xenopus laevis eggs were obtained as described by Newport and Kirschner, Cell (1982) 30:675-686, and were washed twice with MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.8, 0.1 mM EDTA) and dejellied in 2% cysteine. The eggs were then washed twice with MMR/4 and activated by electric shock (5 sec, 12 V); incubated 3 hours at room temperature, followed by 2 washed with 160 mM β-glycerophosphate, 2 mM EGTA, 10 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 25 μg/ml creatine-phosphokinase, 5 mM phosphocreatine, pH 7.5; and then crushed by pipetting and centrifuged for 10 minutes at 10,000 rpm. The crude extract was collected through the lipid layer and recentrifuged for 30 minutes at 40,000 rpm. The clear extract was aliquoted and stored frozen at -80°C.

The resulting cDNA library was probed by transferring the colonies to replicate nitrocellulose filters, and hybridizing to HAV1 and HAV2 as described above. Using the foregoing probes, and selecting colonies hybridizing to both, 3' proximal cloners were obtained. Further portions of the genome were identified by "walking" techniques. An additional clone was obtained using the 5' terminal PstI/PstI fragment from pHAV1307 as probe (Ticehurst, J., et al, (Supra)).

Four recombinant cDNA inserts designated pHAV16, pHAV1, pHAV8, and pHAV47 were selected and restriction maps prepared. The overlapping regions were confirmed by cross-hybridization studies, and the total length of non-overlapping regions was approximately 7.5 kb, the entire span of the HAV genome. The results of the restriction mapping and overlap are shown in Figure 2. pHAV16, pHAV1, pHAV8 and pHAV47 were depos-

ited with the American Type Culture Collection - (ATCC) on 29 March 1985 and assigned accession numbers 53077, 53078, 53076 and 53079, respectively.

Complete sequencing of the series of four inserts shown in Figure 2 gave the results for the corresponding viral RNA set forth in Figure 1. - (Analysis of the open reading frames associated with this sequence indicated a deletion of 22 nucleotides at position 3530 of the HAV genome; this was corrected by site-directed metagenesis to obtain the sequence shown in Figure 1; the corrected plasmid was designated pHAV1*.)

The 7,478 nucleotide heteropolymeric sequence is followed by a polyA tract of undetermined length. A single open reading frame starts with an AUG triplet at nucleotide 734 and terminates with a UGA codon at nucleotide 7,415. Thus, the polyprotein encoded is 2227 amino acids and has a molecular weight of 251,940 daltons. The deduced genomic RNA sequence in Figure 1 shows the 5' non-coding region required for linkage to a tyrosine residue on a viral protein, and the first 75 nucleotides of the sequence can be arranged in the highly stable secondary structure comprising stems and loops known from other picornavirus RNA sequences. Also, the 5' terminal portion contains long U+C repeats, as is generally observed in other picornaviruses.

D.2. Construction of Expression Vectors and Expression of HAV Sequences in E. coli

Four bacterial expression plasmids were constructed which directed the simultaneous synthesis of human superoxide dismutase (SOD) (Hallewell, et al, Nucleic Acids Res (1985) 13:2017 and also portions of all of the hepatitis A capsid precursor protein, P1 (Najarian, et al, Proc Natl Acad Sci - (USA) (1985) 82 :2627. Three of the plasmids synthesized part or all of the P1 fused to the carboxy terminus of SOD. The fourth plasmid also generated a fused mRNA for SOD and P1 gene sequences but contained a sequence between the SOD and P1 genes which provided a stop codon for translation termination of SOD along with a ribosome binding site for reinitiation of translation at the methionine codon thought to be the natural initiator codon of P1. The constructions are shown - schematically in Figure 3.

The expression plasmids were based on the tac promoter driven expression plasmid pSOD16 of Hallewell, et al, (supra). Plasmid pSOD16cf2 was generated from pSOD16 by replacement of a portion of the carboxy terminal coding region of the SOD gene and downstream polylinker sequence through the EcoRI site by the new polylinker sequence

5' GATCGCCATGGGTACCCGGGTCGACTAAATGACTAG 3'

3' CGGTACCCATGGGCCCAGCTGATTTACTGATCTTAA 5'

The substitution of this polylinker sequence results in the removal of the natural carboxy terminal Gln of SOD.

pSODHAV2-2

A 1390 base pair BglII(repair)/XmnI fragment isolated from a digest of pHAV8116SV7d (see ¶ D.4) was cloned into the SmaI site of pSOD16cf2. DNA of a resulting clone having the HAV insert in the proper orientation was digested with KpnI, the 3' overhang was removed with T4 DNA polymerase, then digested the HindIII, and this site was filled in with reverse transcriptase. The resultant linear, blunt ended DNA was religated and transformed into E. coli strain D1210 (Salder, et al, Gene (1980) 8:279-300) to obtain pSODHAV2-2.

pSODHAV2-2 was transformed into E. coliRRIΔM15 (Ruther, U., Nucleic Acids Res - (1982) 10 :5765-5772) for expression to obtain a fusion protein of SOD with the natural SOD carboxy terminal Gln replaced by Met-Glu followed by HAV sequence beginning with the Leu encoded at HAV nucleotide 2072 of the VP3 capsid protein and extending through VP1 to the Val encoded at nucleotide 3201 and terminated with the sequence Gly-Arg-Leu-Asn-Asp encoded by the polylinker.

pSODHAVP1/1

pSODHAVP1/1 contains, in addition to the sequences in pSODHAV2-2, the remainder of VP3 and the carboxy half of VP2. pSODHAV2-2 was digested with NcoI (5' to the HAV sequences) and with SacI, which cuts within the VP1 sequence. The 3.4 kb fragment containing all the SOD sequences and a part of the VP1 sequences was gel isolated, ligated to a 1806 bp NcoI/SacI fragment isolated from pHAV81167d (see ¶ D.4) and transformed into E. coli strain D1210 to obtain pSODHAVP1/1.

When expressed in E. coli strain RRIΔM15, SODHAVP1/1 generated a fusion protein of SOD with HAV sequences starting at the Val of VP2 encoded at nucleotide 1182 through Val of VP1 encoded at nucleotide 3201.

pSODHAV9-1

pSODHAV9-1 was derived from pSODHAVP1/1 and contains the additional half of VP2 to generate the full P1 precursor sequence. A 438 bp BstXI fragment from PHAV47 (see ¶ D.1) containing the 5' end of VP2 was gel isolated and ligated to the single stranded synthetic DNA oligomer,

5'-
ATGAATATGTCCAAACAAGGAATTTTCCAGACTG-
3'

via the BstXI overhang at the 3' end of the 438 bp fragment. The single stranded sequence corresponds to the coding strand of the HAV genome from the extreme 5' end of the P1 precursor. The oligomer was phosphorylated at its 5' end and the strand complementary to the oligomer was synthesized with reverse transcriptase using the 3' hydroxyl of the BstXI overhang of the 438 bp fragment as primer. The resulting fragment was then digested with NcoI, and the NcoI/blunt fragment including the added 30 base pairs of double stranded oligomer, was gel isolated and ligated to pSODHAVP1/1, which had been previously digested with NcoI, at room temperature for 2 hr. The reaction was diluted, and the four dNTPs, Klenow, and additional ligase added to circularize the DNA. The ligation mixture was transformed into E. coli RRIΔM15 to obtain pSODHAV9-1 which contains HAV nucleotides 734 to 3201 (VP2, VP3, VP1).

pSODHAV10-5

pSODHAV10-5 was constructed in a manner analogous to that described above for pSODHAV9-1 except that the synthetic DNA oligomer

5' GATAGAGGAATTATAAGATGAATATGTC-
CAAACAAGGAATTTTCCAGACTG 3'

was used. The oligomer provides for stop codon and a ribosome binding site, in addition to HAV sequences, downstream. pSODHAV10-5 when expressed results in free SOD with the natural terminal Gln replaced with the sequence Met-Ile-Glu-Glu-Leu, and also free HAV P1 lacking its 13 carboxy terminal amino acids, which were replaced with Gly-Arg-Leu-Asn-Asp.

Expression

E. coli strain RRIΔM15, Ruther, U. (supra), cells were transformed with the plasmids described above and single colony transformants were grown overnight at 37°C in 2 ml L-broth plus 100 μg/ml ampicillin. Glycerol (50%) stocks of these cultures were prepared and stored at -20°C.

For protein expression analysis, overnight cultures, in medium as above, were begun from glycerol stocks. These cultures were diluted 1/100 into the same medium and grown at 37°C to an OD_{650} of 0.5-0.8. Uninduced controls were prepared by diluting a 1 ml portion of the growing culture with an equal volume of the same medium. Tac promoter-driven protein synthesis was induced either by diluting of 1 ml of growing culture with medium plus 2 mM IPTG (Isopropyl β-thiogalactopyranoside) or by addition of 1/100 volume of 100 mM IPTG to larger volume cultures, in either case, to give 1 mM IPTG, for 3-4 hr at 37°C. Large scale cultures had 50 ml medium in 500 ml flasks or 250 ml medium in liter flasks.

Cells were lysed in the presence of SDS and DTT for analysis on denaturing polacrylamide gels (Leammli, Nature (1970) 277:680) or with glass beads for analysis on non-denaturing polyacrylamide gels.

Expression was detected by the appearance of (a) protein band(s) of expected size present in induced sample lysates but not in uninduced samples; induced proteins were detected at their respective, expected molecular weights for all plasmids.

The HAV component of fusions and the free P1 of pSODHAV10-5 were detected by Coomassie staining and by specific reaction with anti-HAV antibodies. The SOD component of fusions was detected by Coomassie staining and by anti-SOD antibodies; free SOD was detected both by Coomassie stain and by enzymatic activity in native gels. Gels were either stained with Coomassie blue or subjected to Western analysis after electroblotting onto nitrocellulose filters (Towbin et al, Proc Natl Acad Sci (USA) (1979) 76:3450); blots were preincubated with 0.3% Tween 20 in PBS for 1 hr at room temperature or overnight at 4°C, treated with anti-HAV or anti SOD at 1:100 dilution, and incubated for 1 hr at room temperature. (Rabbit anti-HAV was prepared using partially disrupted HAV virions. Anti-human SOD was prepared by sequential exposure of rabbits to purified recombinant human SOD produced in yeast (see EPO 138 111).) Blots were washed extensively in 0.3% Tween/PBS and incubated for 1 hr at room temperature with a 1:200 dilution of horseradish

peroxidase conjugated goat anti-rabbit-IgG antibody, then washed and treated with Bio-Rad HRP color reagent at 0.5 mg/ml in 8 mM Tris HCl pH 7.5, 16 mM NaCl, 0.015% H_2O_2, 16% methanol.

SOD activity in non-denaturing gels was identified by the method of Beauchamp, C., et al, Anal Biochem (1971) 44:276. Briefly, gels were soaked in the absence of light, first in 2.5 mM nitro blue tetrazolium for 20 min at room temperature, then in 36 mM KPO_4, pH 7.8, 29 mM TEMED, 28 µM riboflavin for 15 min at room temperature. Buffer was removed and the gel was illuminated for 15 min from below using a fluorescent light box. The results of these analyses are shown in Table 1.

Table 1

Plasmid	MW (induced) Calculated	Observed	Coomassie staining (PAGE)	Western with anti-HAV antibody	SOD activity
pSODJAV2-2	59,120	58,880	+	+	nt
pSODHAVP1/1	92,160	88,820	+	+	-
pSODHAV9-1	108,910	105,900	-	+	nt
pSODHAV10-5	92,970(P1)	84,140	-	+	-
	16,440(SOD)	20,650	+	-	+

nt=not tested

Expressed products were approximately 1-3% of total cell protein for pSODHAV2-2 and pSODHAV1/1 and about 50-fold lower for pSODHAV9-1 and pSODHAV10-5.

The SODHAV2-2 and SODHAVP1/1 fusion proteins were purifed on preparatory SDS-acrylamide gels, located by Coomassie staining, and electroeluted. These purified proteins were injected into mice and the resulting sera were tested for reactivity with HAV virus on Western blots and in ELISA assays. Mouse antisera against the SODHAV2-2 react with a 34 kd protein from HAV virus on Western blocks that appears to be HAV-VP1 and was therefore also designated anti-SOD-VP1. In a capture ELISA both mouse anti-SODHAV2-2 and anti-SODHAV1/1 react with HAV virus.

In the ELISA assay, microtiter wells were coated overnight with IgG from an HAV seropositive patient. The coated wells were washed and incubated 1 hr with purified HAV. Dilutions of the mouse sera were the incubated in the well for 1 hr. The wells were then incuated 1 hr with peroxidase conjugated goat anti-mouse Ig followed by reaction with the peroxidase substrate 2,2'azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and H_2O_2, and the plates were read on an ELISA reader at 414 nm; the titer is expressed as the reciprocal of the dilution that gives 50% of the maximum absorbance. The results are as follows:

Control Mouse a	< 10
Control Mouse b	< 10
Mouse Anti-SOD-VP1 a	308
Mouse Anti-SOD-VP1 b	158
Mouse Anti-SOD-P1 a	183
Mouse Anti-SOD-P1 b	160
Mouse Anti-SOD-P1 c	30
Mouse Anti-SOD-P1 d	42

In additional assays, 20 ng of SODHAV2-2 and SODHAVP1/1 were spotted onto nitrocellulose strips, and the strips were then reacted with two HAV seropositive human sera and two seronegative human sera. The strips were washed and reacted with peroxidase conjugated goat anti-human IgG, and then reacted with the peroxidase substrate 4-chloro-1-naphthol to visualize the binding of the peroxidase conjugated antibody. The results showed that the SODHAVP1/1 reacted with both the seropositive samples and neither of the seronegative samples; the SODHAV2-2 reacted with one of two seropositive samples and neither seronegative sample.

In another assay, SODHAVP1/1 was used as the coating antigen in an ELISA assay with HAV seronegative and HAV seropositive human and chimpanzee sera. All four positive sera showed a greater reaction with the SODHAVP1/1 than did the four negative sera. In this assay, microtiter wells were coated with solution of 5 µg/ml of SODHAVP1/1. The coated wells were then reacted with a 1:50 dilution of the various chimp and human sera for 1 hr, and were then incubated with peroxidase conjugated goat anti-human IgG (which cross-reacts with chimp IgG). The wells were finally incubated with ABTS and H_2O_2, and read on an ELISA reader at 414 nm. The results are given in absorbance units.

Serum	HAV Reactive	
Human-1	–	0.211
Human-2	–	0.346
Chimp-1	–	0.459
Chimp-2	–	0.100
Human-3	+	0.787
Human-4	+	0.483
Human-5	+	0.788
Chimp-3	+	1.224

D.3. Construction of Amino Acid Sequences Corresponding to Putative Epitopes

Three short amino acid sequences were synthesized based on analysis of the deduced amino acid sequence of HAV and comparison with the known epitopes of polio virus protein. The hydrophilicity and structural characteristics as a function of position for both the polio polypeptide and the HAV polypeptide were compared with the aid of a computer-based analysis. "Peaks" representing putative areas of structural homology have been identified with corresponding Roman numerals and are shown in Figure 4.

Areas III and V of the polio virus polypeptide represent major antigenic sites of VP1 (Minor et al, Nature (1983) 301:674-679; Evans et al, Nature (1983) 304:459-462). Based on this information, and upon the homology between HAV and polio, two polypeptides comprising sequences that give rise to the corresponding HAV peaks Va and IIIa (Figure 4) were synthesized. A third polypeptide corresponding to an HAV region with characteristics similar to those of Va and IIIa but with no homologous region in the polio sequence was also synthesized.

The three sequences constructed are as follows, where the numbers correspond to the positions designated in the deduced sequence of Figure 1.

Peak IIIa:

561 565

Pro-Glu-Leu-Lys-Pro-Gly-Glu-Ser-Arg-

570 575 576

His-Thr- Ser-Asp-His-Met-Ser-Cys;

Peak Va

591 595

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-

600 602

Thr-Phe-Pro-Cys;

No Polio Homology

606 610 615

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-Pro-

618

Ser-Thr-Cys.

Each of these sequences ends with a C-terminal cysteine for use in conjugation by a covalent bond to a carrier protein of sufficient size to confer immunogenicity of the peptide epitope.

The synthesized proteins were conjugated to the carrier protein, keyhole limpet hemocyanin - (KLH) by disulfide linkage through the cysteine of the peptide using m-maleimido-benzoyl-N-hydroxysuccinimide ester (MBS) as the coupling reagent - (Liu et al, Biochem (1979) 18:690-697; Green, N., et al, Cell (1982) 28:477-487). Alternatively, the peptides were coupled to KLH using glutaraldehyde (0.05-0.3%).

The conjugated peptides were injected into rabbits. Sera obtained after 31-100 days contained antibodies against the injected conjugates as assayed by ELISA. Western blots using the antisera against gels run on tissue culture-produced HAV show immunoprecipitates for the antisera produced in response to the protein corresponding to peak Va and to the protein with no polio homology. Immunoprecipitation was observed with the bands corresponding to VP1 protein.

D.4. Preparation of Non-Infectious (Deleted) Virions

Deleted virions can be prepared using expression vectors compatible with procaryotic, yeast, and mammalian cells.

The P1 precursor of several picornaviruses synthesized in vitro can be processed into individual virion proteins which then assemble into immunoreactive virion-like particles (Nicklin, et al, Biotechnology (1986) 4:33; Palmenberg, et al, J. Virol (1979) 32:770; Shih, et al, Proc Natl Acad Sci (USA) (1978) 75:5807; Hanecak, et al, Proc Natl Acad Sci (USA) (1982) 79:3973; Grubman, et al, J Virol (1985) 56:120).

To obtain a similar effect in vivo, the E. coli transfected with pSODHAV10-5, which produces the P1 precursor (see ¶D.2) can be modified to produce the virus encoded proteinase (3C) along with the P1 protein. The resulting combination of P1 precursor coexpressed with proteinase will yield non-infectious, assembled virion-like particles.

Similar constructs expressing the P1 precursor and proteinase can be produced in yeast and mammalian cells. For production of non-infectious virion-like particles, mammalian cells can be transfected with the HAV genome under the control of appropriate regulatory elements. By deleting the 3' end portion of the genome beyond the sequences encoding 3C (ie., the 3D region) replicase gene is removed, thereby crippling the replication ability of the virus. The transfected cells will produce all virion proteins from the P1 and P2 region in addition to 3A, 3B and 3C. This is sufficient to generate virion-like particles, but will not generate infectious virus.

For example, an expression vector compatible with mammalian cells pHAV8161-SV7d, is constructed as shown in Figure 5. The HAV coding sequences are provided by digestion of pHAV1*, pHAV8, and pHAV16 (¶D.1). The mammalian replication and control systems are provided by pSV7d (see below), which is a pBR322 derivative modified to contain the SV40 origin of replication, early promoter and polyadenylation site. It also has a polylinker with several restriction sites downstream from the SV40 promoter, as well as TAA stop codons in all three reading frames downstream from the polylinker.

In more detail, pSV7d was constructed as follows: the 400 bp BamHI/HindIII fragment containing the SV40 origin of replication and early promoter was excised from pSVgtl (Mulligan et al, Mol Cell Biol (1981) 1:854-864) and purified. The 240 bp SV40 BclI/BamHI fragment containing SV40 polyA addition sites was excised from pSV2/dhfr - (Subramani et al, J Mol Cell Biol (1981) 1:854-864) and purified. The fragments were fused through the linker:

```
                Stop Codons
                 1   2   3

5'-AGCTAGATCTCCCGGGTCTAGATAAGTAAT-3'
       TCTAGAGGGCCCAGATCTATTCATTACTAG

HindIII  BglII SmaI  XbaI       BclI overhang
```

which contains the restriction sites shown and stop codons in all three reading frames. The resulting 670 bp. fragment, containing SV40 origin of replication, SV40 early promoter, polylinker with stop codons and SV40 polyadenylation site was closed into the BamHI site of pML, a pBR322 derivative with an about 2.5 kb deletion (Lusky and Botchan, Cell (1984) 36:391), to yield pSV6. The EcoRI and EcoRV sites in the pML sequences of pSV6 were eliminated by digestion with EcoRI and EcoRV, treatment with Bal31 nuclease to remove about 200 bp per end, and religation to yield pSV7a. (The Bal31 resection also eliminated one BamHI restriction site flanking the SV40 region approximately 200 bp away from the EcoRV site. To eliminate the other BamHI site flanking the SV40 region, pSV7a was digested with NruI, which cuts in the pML sequence upstream from the origin of replication, and recircularized by blunt end ligation to yield pSV7b.

pSV7c and pSV7d represent successive polylinker replacements. First, pSV7b was digested with StuI and XbaI, and the linker:

```
BglII EcoRI  SmaI   KpnI XbaI

5'-AGATCTCGAATTCCCCGGGGGTACCT
   TCTAGAGCTTAAGGGGCCCCCATGGAGATC
```

was ligated into the vector to yield pSV7c.

pSV7c was digested with BglII and XbaI, and the linker:

```
BglII EcoRI   SmaI XbaI  BamHI SalI

5'-GATCTCGAATTCCCCGGGTCTAGAGGATCCGTCGAC
      AGCTTAAGGGGCCCAGATCTCCTAGGCACGTGATC
```

was ligated to yield pSV7d.

In the construction of pHAV8161-SV7d, pHAV161 (supra) was digested with BamHI and SalI to release the 4.2 kb fragment encoding a portion of the HAV sequence. pHAV8 was digested with SalI and BamHI to provide the coding sequences upstream of the SalI site in the pHAV161 insert. These two fragments were ligated into the BamHI site of pSV7d. The resulting vector pHAV8161-SV7d places the ligated HAV sequences encoding amino acid 1-2076 under control of the SV40 promotor and directly behind the vector provided stop codon, as shown in Figure 8.

pHAV8161-SV7d may be transfected into COS cells and transient expression tested by immunofluorescence using human convalescent serum for HAV. Plasmid DNA from successful COS transformants may then be used to construct vectors for stable expression.

D.5. Hybrid Particle HAV Immunogens

U.S. Serial No. 650,323, filed 12 April 1984 and assigned to the same assignee is incorporated herein by reference. This application describes the construction of hybrid particles of hepatitis B surface antigen (HBsAg) containing inserts of foreign immunogens into a pre-surface (pre-S) coding portion in reading frame with the codons for HBsAg. Plasmid pDC101, described therein contains a portion of the pre-S/HBsAg gene, including 55 codons of the pre-S region, in a GAPDH controlled expression cassette cloned into the BamHI site of pBR322 derivative. The incorporated application describes the insertion of desired immunogens, such as the gD (glycoprotein D) antigenic site into a unique EcoRI site present in the pre-S region of pDC101 to give the hybrid plasmid pDC103. Similarly, in accordance with the present invention, desired epitopes derived from the HAV genome may be provided with suitable EcoRI linkers and inserted in proper reading frames into the EcoRI site of pDC101, or used to replace the gD codons in the pDC103 hybrid. pDC103 is deposited with ATCC and has accession no. 20726.

The construction of pDC-104, a yeast expression vector for a hybrid gene containing the sequence encoding HAV-VP1 epitope shown in Figure 6 was as follows. The HindIII/SalI fragment obtained from HAV1307 (and containing codons for amino acids 445-657 of the VP1 region) was provided with synthetic EcoRI linkers and ligated into the EcoRI site of pDC103 in place of the gD sequences. The resultant was transformed into yeast host S. carlsbergensis 2150-2-3 and the cultured hosts harvested. The hybrid HBV/HAV particle is detected in lysates of the yeast cells by using immobilized HBV antisera to capture the antigen and a second soluble antiserum to HAV labeled with horseradish peroxidase. Alternatively, bound anti-HAV antisera can be used to capture the particle, followed by a second labeled anti-HBV antiserum.

pWG-1, a yeast expression vector containing the coding sequence for the hybrid gene harboring the VP-1 neutralizing epitope in the particle-forming portion of the HBsAg-encoding gene is constructed similarly (see Figure 7). The DNA corresponding to the HAV epitope of the following sequence:

```
1   GATCCAGTTTTAGCAAAGAAGGTACCTGAGACATTTCCTGAATTGAAGCCTGGAGAGTCC
        GTCAAAATCGTTTCTTCCATGGACTCTGTAAAGGACTTAACTTCGGACCTCTCAGG

61  AGACATACATCAGATCACATGTCTATTTATAAATTCATGGGAAGGTCTCATTTTTTGTGC
    TCTGTATGTAGTCTAGTGTACAGATAAATATTTAAGTACCCTTCCAGAGTAAAAAACACG

121 ACTTTTACTTTCAATTCAAATAATAAAGAGTACACATTTCCAATCG
    TGAAAATGAAAGTTAAGTTTATTATTTCTCATGTGTAAAGGTTAGCCTAG
```

codes for amino acids 792-848 and is synthesized using a commercially available automated oligonucleotide synthesizer. The resulting synthetic oligomer, containing BamHI sites, is then cloned into the BglII sites of the host vector pDC101, described in U.S. 650,323 (supra), replacing a 150 bp fragment of the HBsAg coding for a dominant epitope. The resulting vector pWG-1 is transformed into S. carlsbergensis as above, and the transformants selected for LEU+. Successful transformants are cultured and grown, and the modified HBsAg particles recovered from the medium, and their bifunctional character confirmed as above.

Hybrid particle immunogens are thus prepared using fused coding sequences for HBsAg and HAV and provide enhanced immunogenicity for the HAV epitopes.

Claims

1. A nucleotide sequence substantially identical with that of the HAV genome as represented in Figure 1.

2. A nucleotide sequence encoding a viral polypep-

tide substantially identical with that encoded by the HAV genomic sequence shown in Figure 1.

3. A recombinant expression system comprising a coding portion derived from the sequence of claim 1 operably linked to a control sequence compatible with a desired host.

4. Recombinant host cells transformed with the expression system of claim 3.

5. Protein produced by the cells of claim 4.

6. The system of claim 3 which further includes, contiguous to said coding portion, and in reading frame therewith, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.

7. The system of claim 3 which further includes, upstream of said coding portion, and separated by a stop codon, a fused nucleotide sequence encoding a non-HAV encoded protein or portion thereof.

8. The system of claim 6 or 7 wherein the fused DNA sequence encodes human superoxide dismutase or portion thereof.

9. Recombinant host cells transformed with the system of claim 6 or 7.

10. Protein produced by the cells of claim 9.

11. The system of claim 3 wherein the coding portion is derived from the nucleotide sequence of claim 1 between nucleotide positions selected from the group consisting of 2072-3201; 1182-3201; and 734-3201.

12. A polypeptide of the sequence selected from the group consisting of:

Pro-Gly-Leu-Lys-Pro-Gly-Glu-Ser-Arg-His-Thr-Ser-Asp-His-Met-Ser-Cys;

Thr-Phe-Asn-Ser-Asn-Asn-Lys-Glu-Tyr-Tyr-Phe-Pro-Cys; and

Ser-Ser-Thr-Ser-Asn-Pro-Pro-His-Gly-Leu-ProSer-Thr-Cys;

wherein said polypeptide is optionally covalently linked to a carrier protein.

13. A particle immunogenic against HAV infection which particle comprises a polypeptide having an amino acid sequence capable of forming a particle when said sequence is produced in a eucaryotic host, and an epitope of HAV.

14. The particle of claim 13 wherein the particle forming amino acid sequence is derived from hepatitis, and the epitope is derived from amino acids 445-657 or 792-848 of the HAV polypeptide sequence.

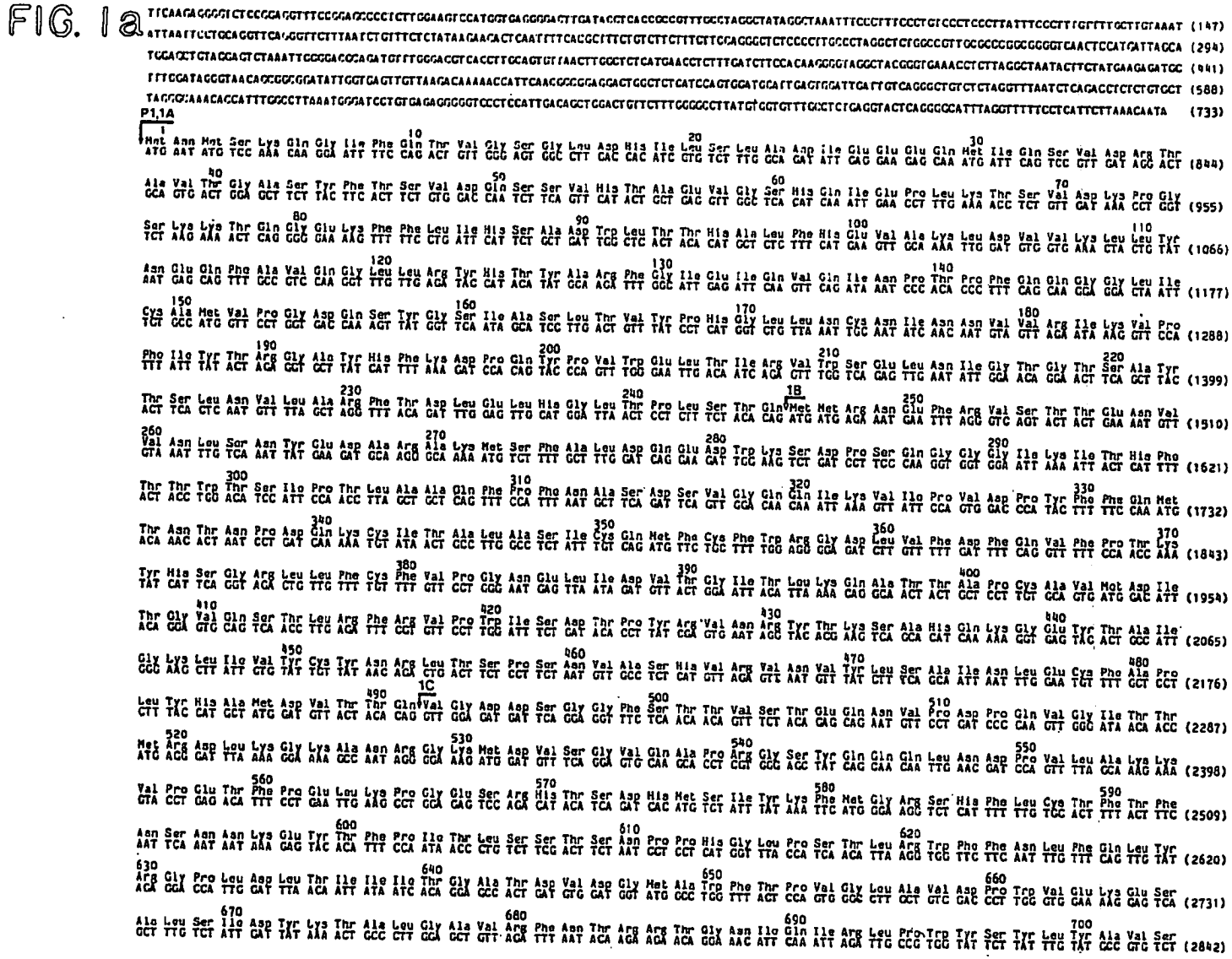
15. An oligonucleotide useful for detecting the presence of HAV in a biological sample which comprises a DNA sequence derived from the nucleotide sequence in Figure 1.

16. A vaccine effective against hepatitis A virus which comprises a polypeptide derived from the polypeptide sequence of HAV.

17. An expression system for a deleted virion which comprises a nucleotide sequence encoding the P1 precursor protein operably linked to suitable control sequences, and transformed into a host cell capable of producing a protein effective in cleaving said P1 precursor to obtain a deleted virion.

18. An expression system which comprises a nucleotide sequence encoding a protein selected from the group consisting of VP1, VP2 and VP3 operably linked to suitable control sequences.

FIG. 1a



0 199 480

FIG. 1b

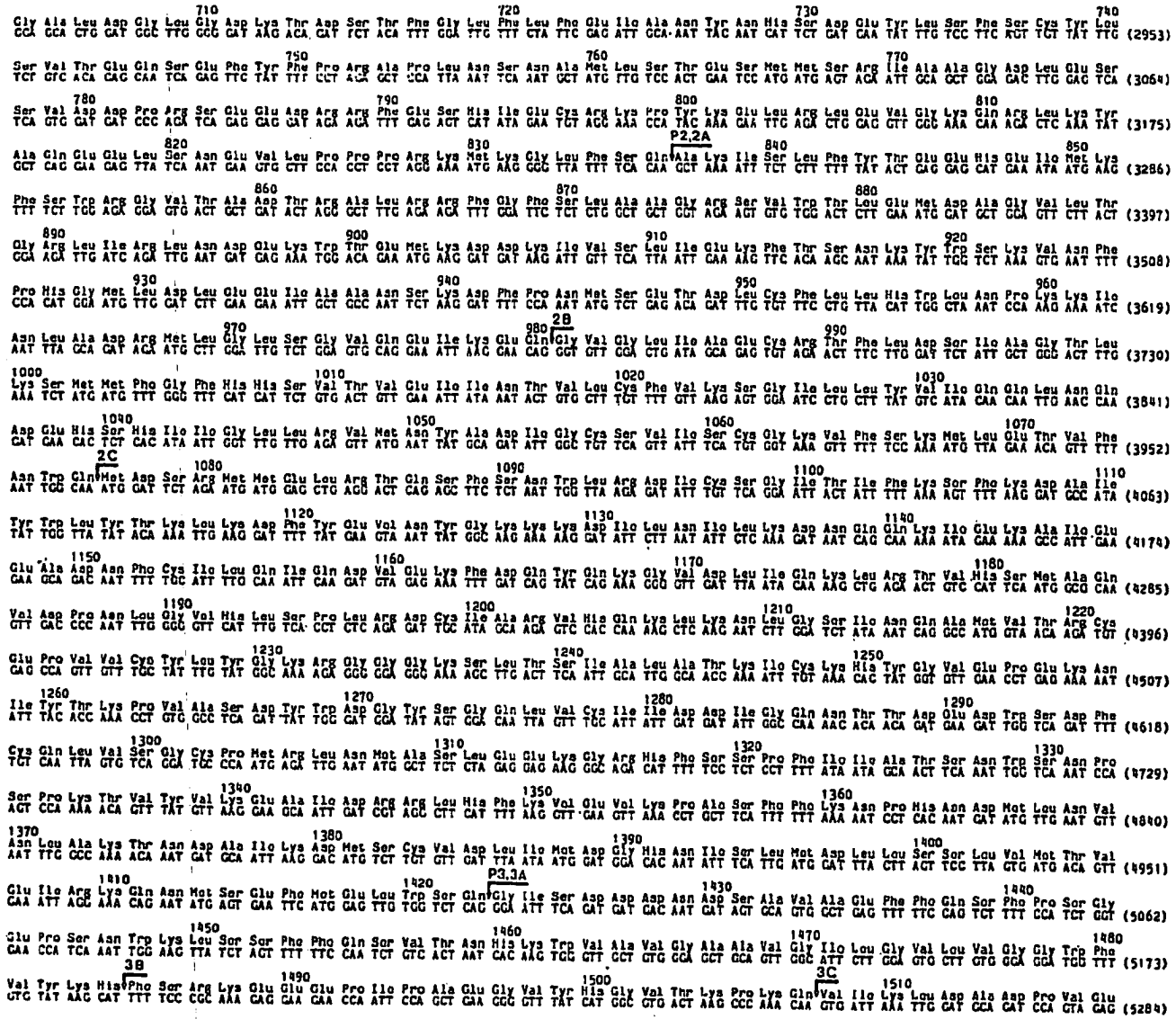

710 720 730 740
Gly Ala Leu Asp Gly Leu Gly Asp Lys Thr Asp Ser Thr Phe Gly Leu Phe Glu Ile Ala Asn Tyr Asn His Ser Asp Glu Tyr Leu Ser Phe Ser Cys Tyr Leu (2953)
750 760 770
Ser Val Thr Glu Gln Ser Glu Phe Tyr Phe Pro Arg Ala Pro Leu Asn Ser Asn Ala Met Leu Ser Thr Glu Ser Met Met Ser Arg Ile Ala Ala Gly Asp Leu Glu Ser (3064)
780 790 800 810
Ser Val Asp Asp Pro Arg Ser Glu Glu Asp Arg Arg Phe Glu Ser His Ile Glu Cys Arg Lys Pro Tyr Lys Glu Leu Arg Leu Glu Val Gly Lys Gln Arg Leu Lys Tyr (3175)
820 830 840 850
Ala Gln Glu Glu Leu Ser Asn Glu Val Leu Pro Pro Pro Arg Lys Met Lys Gly Leu Phe Ser Gln Ala Lys Ile Ser Leu Phe Tyr Thr Glu Glu His Glu Ile Met Lys (3286)
860 870 880
Phe Ser Trp Arg Gly Val Thr Ala Asp Thr Arg Ala Leu Arg Arg Phe Gly Phe Ser Leu Ala Ala Gly Arg Ser Val Trp Thr Leu Glu Met Asp Ala Gly Val Leu Thr (3397)
890 900 910 920
Gly Arg Leu Ile Arg Leu Asn Asp Glu Lys Trp Thr Glu Met Lys Asp Asp Lys Ile Val Ser Leu Ile Glu Lys Phe Thr Ser Asn Lys Tyr Trp Ser Lys Val Asn Phe (3508)
930 940 950 960
Pro His Gly Met Leu Asp Leu Glu Glu Ile Ala Ala Asn Ser Lys Asp Phe Pro Asn Met Ser Glu Thr Asp Leu Cys Phe Leu Leu His Trp Leu Asn Pro Lys Lys Ile (3619)
970 980 990
Asn Leu Ala Asp Arg Met Leu Gly Leu Ser Gly Val Gln Glu Ile Lys Glu Gln Gly Val Gly Leu Ile Ala Glu Cys Arg Thr Phe Leu Asp Ser Ile Ala Gly Thr Leu (3730)
1000 1010 1020 1030
Lys Ser Met Met Phe Gly Phe His His Ser Val Thr Val Glu Ile Ile Asn Thr Val Leu Cys Phe Val Lys Ser Gly Ile Leu Leu Tyr Val Ile Gln Gln Leu Asn Gln (3841)
1040 1050 1060 1070
Asp Glu His Ser His Ile Ile Gly Leu Leu Arg Val Met Asn Tyr Ala Asp Ile Gly Cys Ser Val Ile Ser Cys Gly Lys Val Phe Ser Lys Met Leu Glu Thr Val Phe (3952)
1080 1090 1100 1110
Asn Trp Gln Met Asp Ser Arg Met Met Glu Leu Arg Thr Gln Ser Phe Ser Asn Trp Leu Arg Asp Ile Cys Ser Gly Ile Thr Ile Phe Lys Ser Phe Lys Asp Ala Ile (4063)
1120 1130 1140
Tyr Trp Leu Tyr Thr Lys Leu Lys Asp Phe Tyr Glu Val Asn Tyr Gly Lys Lys Lys Asp Ile Leu Asn Ile Leu Lys Asp Asn Gln Gln Lys Ile Glu Lys Ala Ile Glu (4174)
1150 1160 1170 1180
Glu Ala Asp Asn Phe Cys Ile Leu Gln Ile Gln Asp Val Glu Lys Phe Asp Gln Tyr Gln Lys Gly Val Asp Leu Ile Gln Lys Leu Arg Thr Val His Ser Met Ala Gln (4285)
1190 1200 1210 1220
Val Asp Pro Asn Leu Gly Val His Leu Ser Pro Leu Arg Asp Cys Ile Ala Arg Val His Gln Lys Leu Lys Asn Leu Gly Ser Ile Asn Gln Ala Met Val Thr Arg Cys (4396)
1230 1240 1250
Glu Pro Val Val Cys Tyr Leu Tyr Gly Lys Arg Gly Gly Gly Lys Ser Leu Thr Ser Ile Ala Leu Ala Thr Lys Ile Cys Lys His Tyr Gly Val Glu Pro Glu Lys Asn (4507)
1260 1270 1280 1290
Ile Tyr Thr Lys Pro Val Ala Ser Asp Tyr Trp Asp Gly Tyr Ser Gly Gln Leu Val Cys Ile Ile Asp Asp Ile Gly Gln Asn Thr Thr Asp Glu Asp Trp Ser Asp Phe (4618)
1300 1310 1320 1330
Cys Gln Leu Val Ser Gly Cys Pro Met Arg Leu Asn Met Ala Ser Leu Glu Glu Lys Gly Arg His Phe Ser Ser Pro Phe Ile Ile Ala Thr Ser Asn Trp Ser Asn Pro (4729)
1340 1350 1360
Ser Pro Lys Thr Val Tyr Val Lys Glu Ala Ile Asp Arg Arg Leu His Phe Lys Val Glu Val Lys Pro Ala Ser Phe Phe Lys Asn Pro His Asn Asp Met Leu Asn Val (4840)
1370 1380 1390 1400
Asn Leu Ala Lys Thr Asn Asp Ala Ile Lys Asp Met Ser Cys Val Asp Leu Ile Met Asp Gly His Asn Ile Ser Leu Met Asp Leu Leu Ser Ser Leu Val Met Thr Val (4951)
1410 1420 1430 1440
Glu Ile Arg Lys Gln Asn Met Ser Glu Phe Met Glu Leu Trp Ser Gln Gly Ile Ser Asp Asp Asp Asn Asp Ser Ala Val Ala Glu Phe Phe Gln Ser Phe Pro Ser Gly (5062)
1450 1460 1470 1480
Glu Pro Ser Asn Trp Lys Leu Ser Ser Phe Phe Gln Ser Val Thr Asn His Lys Trp Val Ala Val Gly Ala Ala Val Gly Ile Leu Gly Val Leu Val Gly Gly Trp Phe (5173)
1490 1500 1510
Val Tyr Lys His Phe Ser Arg Lys Glu Glu Glu Pro Ile Pro Ala Glu Gly Val Tyr His Gly Val Thr Lys Pro Lys Gln Val Ile Lys Leu Asp Ala Asp Pro Val Glu (5284)
P2,2A
2B
2C
3B
3C
P3,3A

FIG. 1c

```
                1520                1530                1540                1550
Ser Gln Ser Thr Leu Glu Ile Ala Gly Leu Val Arg Lys Asn Leu Val Gln Phe Gly Val Gly Glu Lys Asn Gly Cys Val Arg Trp Val Met Asn Ala Leu Gly Val Lys (5395)
TCC CAG TCA ACT CTA GAA ATA GCA GGA TTA GTT AGG AAA AAT CTG GTT CAG TTT GGA GTT GGT GAG AAA AAT GGA TGT GTG AGA TGG GTC ATG AAT GCC TTA GGA GTG AAG

                1560                1570                1580                1590
Asp Asp Trp Leu Leu Val Pro Ser His Ala Tyr Lys Phe Glu Lys Asp Tyr Glu Met Met Glu Phe Tyr Phe Asn Arg Gly Gly Thr Tyr Tyr Ser Ile Ser Ala Gly Asn (5506)
GAT GAT TGG TTG TTA GTA CCT TCT CAT GCT TAT AAA TTT GAA AAG GAT TAT GAA ATG ATG GAG TTT TAC TTC AAT AGA GGT GGA ACT TAC TAT TCA ATT TCA GCT GGT AAT

                1600                1610                1620
Val Val Ile Gln Ser Leu Asp Val Gly Phe Gln Asp Val Val Leu Met Lys Val Pro Thr Ile Pro Lys Phe Arg Asp Ile Thr Gln His Phe Ile Lys Lys Gly Asp Val (5617)
GTT GTT ATT CAA TCT TTA GAT GTG GGA TTT CAA GAT GTT GTT TTA ATG AAG GTT CCT ACA ATT CCC AAG TTT AGA GAT ATT ACT CAA CAC TTT ATT AAG AAA GGA GAT GTC

        1630                1640                1650                1660
Pro Arg Ala Leu Asn Arg Leu Ala Thr Leu Val Thr Thr Val Asn Gly Thr Pro Met Leu Ile Ser Glu Gly Pro Leu Lys Met Glu Glu Lys Ala Thr Tyr Val His Lys (5728)
CCT AGA GCC TTA AAT CGC TTG GCA ACA TTA GTG ACA ACC GTT AAT GGA ACT CCT ATG TTA ATT TCT GAG GGA CCA TTA AAG ATG GAA GAA AAA GCC ACT TAT GTT CAT AAG
                                    3D
                1670                1680                1690                1700
Lys Asn Asp Gly Thr Thr Val Asp Leu Thr Val Asp Gln Ala Trp Arg Gly Lys Gly Glu Gly Leu Pro Gly Met Cys Gly Gly Ala Leu Val Ser Ser Asn Gln Ser Ile (5839)
AAG AAT GAT GGT ACT ACA GTT GAT TTG ACT GTA GAT CAG GCA TGG AGA GGA AAA GGT GAA GGT CTT CCT GGA ATG TGT GGT GGG GCC CTA GTG TCA TCA AAT CAG TCC ATA

                1710                1720                1730
Gln Asn Ala Ile Leu Gly Ile His Val Ala Gly Gly Asn Ser Ile Leu Val Ala Lys Leu Val Thr Gln Glu Met Phe Gln Asn Ile Asp Lys Lys Ile Glu Ser Gln Arg (5940)
CAG AAT GCA ATT TTG GGT ATT CAT GTT GCT GGA GGA AAT TCA ATT CTT GTG GCA AAG CTG GTT ACT CAA GAA ATG TTT CAA AAC ATT GAT AAG AAA ATT GAA AGT CAG AGA

        1740                1750                1760                1770
Ile Met Lys Val Glu Phe Thr Gln Cys Ser Met Asn Val Val Ser Lys Thr Leu Phe Arg Lys Ser Pro Ile His His His Ile Asp Lys Thr Met Ile Asn Phe Pro Ala (6051)
ATA ATG AAA GTG GAA TTT ACT CAA TGT TCA ATG AAT GTA GTC TCC AAA ACG CTT TTT AGA AAG AGT CCC ATT CAT CAC CAC ATT GAT AAA ACC ATG ATT AAT TTT CCT GCA

                1780                1790                1800                1810
Ala Met Pro Phe Ser Lys Ala Glu Ile Asp Pro Met Ala Met Met Leu Ser Lys Tyr Ser Leu Pro Ile Val Glu Glu Pro Glu Asp Tyr Lys Glu Ala Ser Val Phe Tyr (6162)
GCT ATG CCT TTC TCT AAA GCT GAA ATT GAT CCA ATG GCT ATG ATG TTG TCC AAA TAT TCA TTA CCT ATT GTG GAG GAA CCA GAG GAT TAC AAG GAA GCT TCA GTT TTT TAT

                1820                1830                1840                1850
Gln Asn Lys Ile Val Gly Lys Thr Gln Leu Val Asp Asp Phe Leu Asp Leu Asp Met Ala Ile Thr Gly Ala Pro Gly Ile Asp Ala Ile Asn Met Asp Ser Ser Pro Gly (6273)
CAA AAC AAA ATA GTA GGC AAG ACT CAG CTA GTT GAT GAC TTT TTA GAT CTT GAT ATG GCT ATT ACA GGG GCT CCA GGC ATT GAT GCT ATC AAT ATG GAT TCA TCT CCT GGG

                1860                1870                1880
Phe Pro Tyr Val Gln Glu Lys Leu Thr Lys Arg Asp Leu Ile Trp Leu Asp Glu Asn Gly Leu Leu Leu Gly Val His Pro Arg Leu Ala Gln Arg Ile Leu Phe Asn Thr (6384)
TTT CCT TAT GTT CAA GAA AAA TTG ACC AAA AGA GAT TTA ATT TGG TTG GAT GAA AAT GGT TTG CTG TTA GGA GTT CAC CCA AGA TTG GCC CAG AGA ATT TTA TTT AAT ACT

        1890                1900                1910                1920
Val Met Met Glu Asn Cys Ser Asp Leu Asp Val Val Phe Thr Thr Cys Pro Lys Asp Glu Leu Arg Pro Leu Glu Lys Val Leu Glu Ser Lys Thr Arg Ala Ile Asp Ala (6495)
GTC ATG ATG GAA AAT TGT TCT GAC TTA GAT GTT GTT TTT ACA ACT TGT CCA AAA GAT GAA TTG AGA CCA TTA GAG AAA GTT TTG GAA TCA AAA ACA AGA GCC ATT GAT GCT

                1930                1940                1950                1960
Cys Pro Leu Asp Tyr Thr Ile Leu Cys Arg Met Tyr Trp Gly Pro Ala Ile Ser Tyr Phe His Leu Asn Pro Gly Phe His Thr Gly Val Ala Ile Gly Ile Asp Pro Asp (6606)
TGT CCT TTG GAT TAT ACA ATT CTA TGT CGA ATG TAT TGG GGT CCA GCT ATC AGT TAT TTC CAT TTG AAT CCA GGG TTT CAC ACA GGT GTT GCT ATT GGC ATA GAT CCT GAT

                1970                1980                1990
Arg Gln Trp Asp Glu Leu Phe Lys Thr Met Ile Arg Phe Gly Asp Val Gly Leu Asp Leu Asp Phe Ser Ala Phe Asp Ala Ser Leu Ser Pro Phe Met Ile Arg Glu Ala (6717)
AGA CAG TGG GAT GAA TTA TTT AAA ACA ATG ATA AGA TTT GGA GAT GTT GGT CTT GAT TTA GAT TTC TCT GCT TTT GAT GCC AGT CTT AGT CCA TTT ATG ATT AGG GAA GCA

        2000                2010                2020                2030
Gly Arg Ile Met Ser Glu Leu Ser Gly Thr Pro Ser His Phe Gly Thr Ala Leu Ile Asn Thr Ile Ile Tyr Ser Lys His Leu Leu Tyr Asn Cys Cys Tyr His Val Cys (6828)
GGT AGA ATC ATG AGT GAA TTA TCT GGA ACA CCA TCT CAT TTT GGA ACA GCT CTT ATC AAT ACT ATC ATT TAT TCT AAA CAT CTG CTG TAC AAC TGT TGT TAT CAT GTT TGT

                2040                2050                2060                2070
Gly Ser Met Pro Ser Gly Ser Pro Cys Thr Ala Leu Leu Asn Ser Ile Ile Asn Asn Ile Asn Leu Tyr Tyr Val Phe Ser Lys Ile Phe Gly Lys Ser Pro Val Phe Phe (6939)
GGT TCA ATG CCT TCT GGG TCT CCT TGC ACA GCT TTG TTG AAT TCA ATT ATT AAT AAT ATT AAT CTG TAT TAT GTG TTT TCT AAA ATA TTT GGA AAG TCT CCA GTT TTC TTT

                2080                2090                2100
Cys Gln Ala Leu Arg Ile Leu Cys Tyr Gly Asp Asp Val Leu Ile Val Phe Ser Arg Asp Val Gln Ile Asp Asn Leu Asp Leu Ile Gly Gln Lys Ile Val Asp Glu Phe (7050)
TGT CAA GCT TTG AGG ATC CTT TGT TAC GGA GAT GAT GTT TTG ATA GTT TTT TCC AGA GAT GTT CAA ATT GAC AAT CTT GAC TTG ATT GGA CAG AAA ATT GTA GAT GAG TTC

        2110                2120                2130                2140
Lys Lys Leu Gly Met Thr Ala Thr Ser Ala Asp Lys Asn Val Pro Gln Leu Lys Pro Val Ser Glu Leu Thr Phe Leu Lys Arg Ser Phe Asn Leu Val Glu Asp Arg Ile (7161)
AAA AAA CTT GGC ATG ACA GCC ACC TCA GCT GAT AAA AAT GTG CCT CAA CTG AAG CCA GTT TCA GAA TTG ACT TTT CTC AAA AGA TCT TTC AAT TTG GTG GAG GAT AGA ATT

                2150                2160                2170                2180
Arg Pro Ala Ile Ser Glu Lys Thr Ile Trp Ser Leu Met Ala Trp Gln Arg Ser Asn Ala Glu Phe Glu Gln Asn Leu Glu Asn Ala Gln Trp Phe Ala Phe Met His Gly (7272)
AGA CCT GCA ATT TCA GAA AAG ACA ATT TGG TCT TTG ATG GCT TGG CAG AGA AGT AAC GCT GAG TTT GAG CAG AAT TTA GAA AAT GCT CAG TGG TTT GCT TTT ATG CAT GGC

                2190                2200                2210                2220
Tyr Glu Phe Tyr Gln Lys Phe Tyr Tyr Phe Val Gln Ser Cys Leu Glu Lys Glu Met Ile Glu Tyr Arg Leu Lys Ser Tyr Asp Trp Trp Arg Met Arg Phe Tyr Asp Gln (7383)
TAT GAG TTC TAT CAG AAA TTT TAT TAT TTT GTT CAG TCC TGT TTG GAG AAA GAG ATG ATA GAA TAT AGA CTT AAA TCT TAT GAT TGG TGG AGA ATG AGA TTT TAT GAC CAG

                2227
Cys Phe Ile Cys Asp Leu Ser OP
TGT TTC ATT TGT GAC CTT TCA TGA TTTGTTTAAACAAATTTTCTTACTCTTTCTGAGGTTTGTTTATTTCTTTTGTCCGCTAACTAAAAAAAAAAAAAAAAA (7484)

Translated Mol. Weight = 251,940
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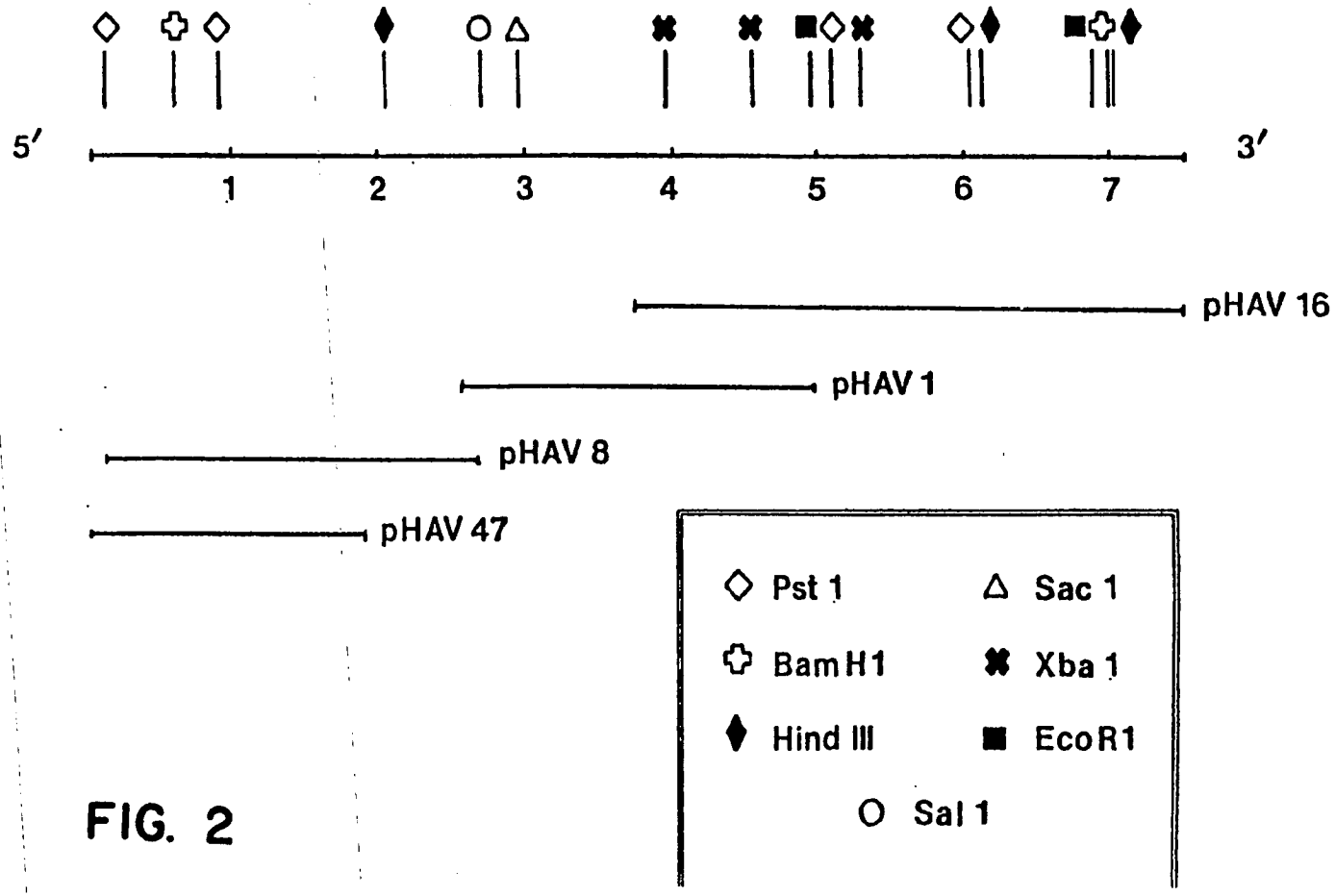
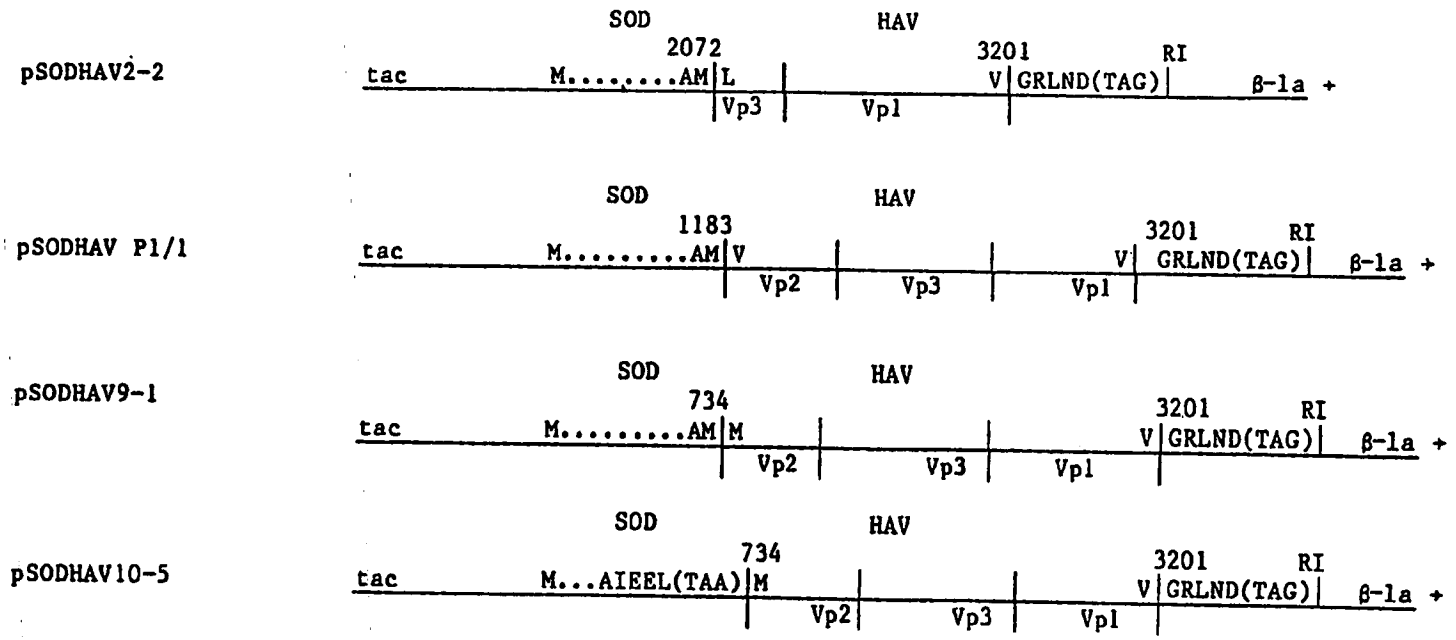

5′
3′
1
2
3
4
5
6
7
pHAV 16
pHAV 1
pHAV 8
pHAV 47
Pst 1
Sac 1
BamH1
Xba 1
Hind III
EcoR1
Sal 1

FIG. 2

FIG. 3 : SCHAEMATIC OF STRUCTURE OF HAV EXPRESSION PLASMIDS



tac= trp/lac promoter/operator.
(TAA), (TAG)= stop codons.
NUMBERS give HAV nucleotides at beginning and end of HAV sequence.
CAPITAL LETTERS give amino acid residues (one letter code)
at start/stops and SOD/HAV/linker junctions.
RI: EcoRI restriction site.

0 199 480

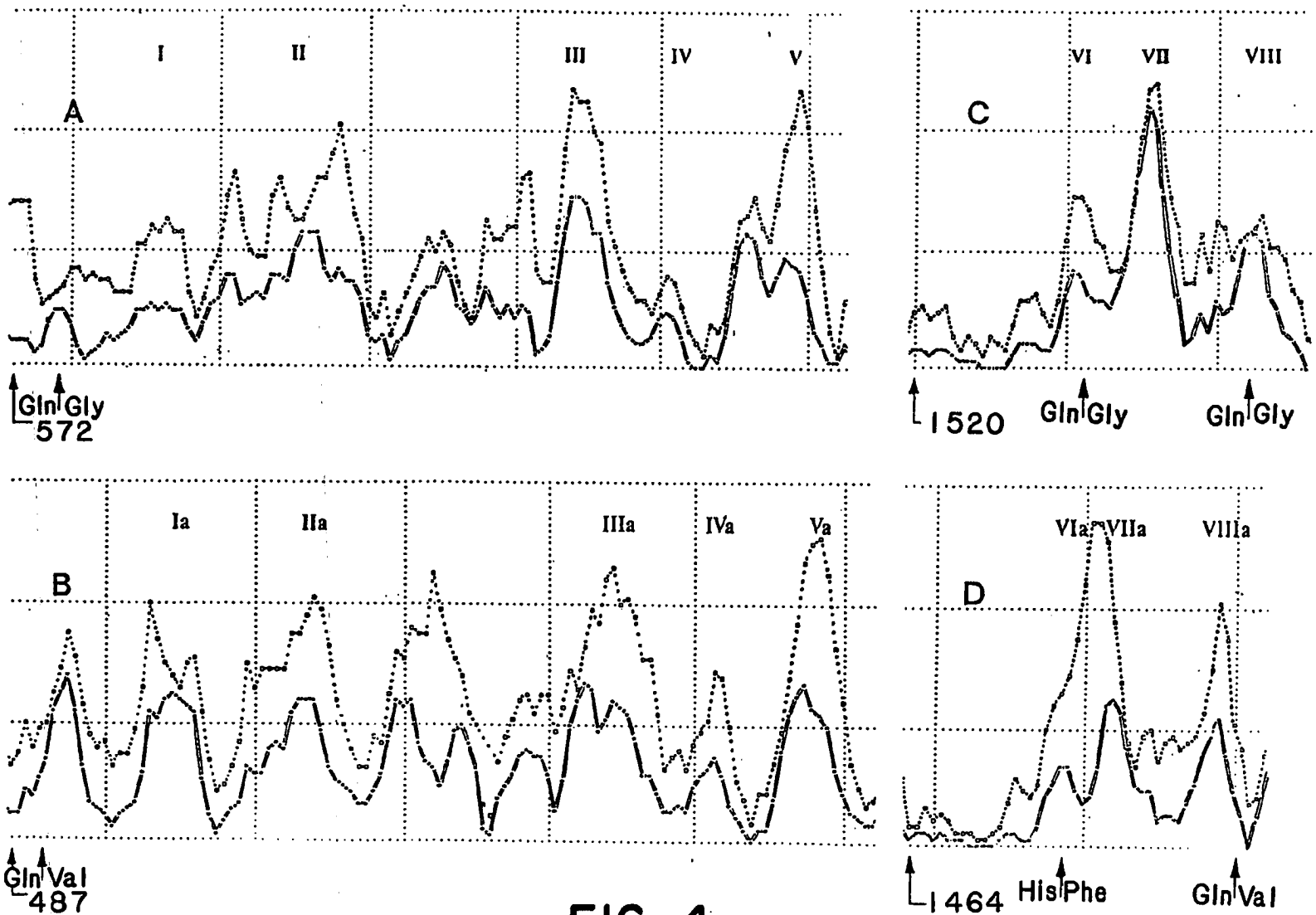
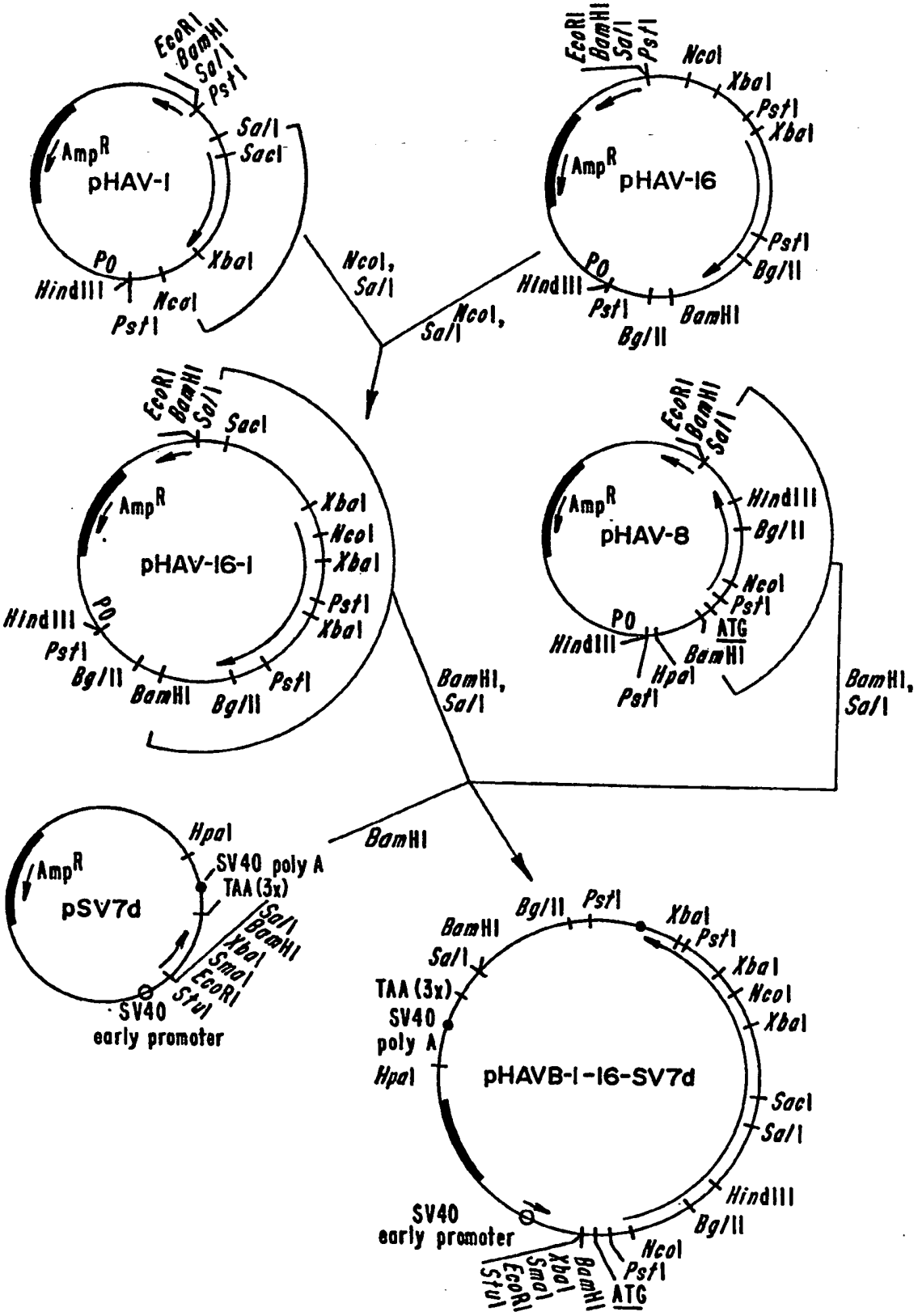


FIG. 4

0 199 480

FIG. 5

Construction of HAV-Mammalian Cell Expression Vectors



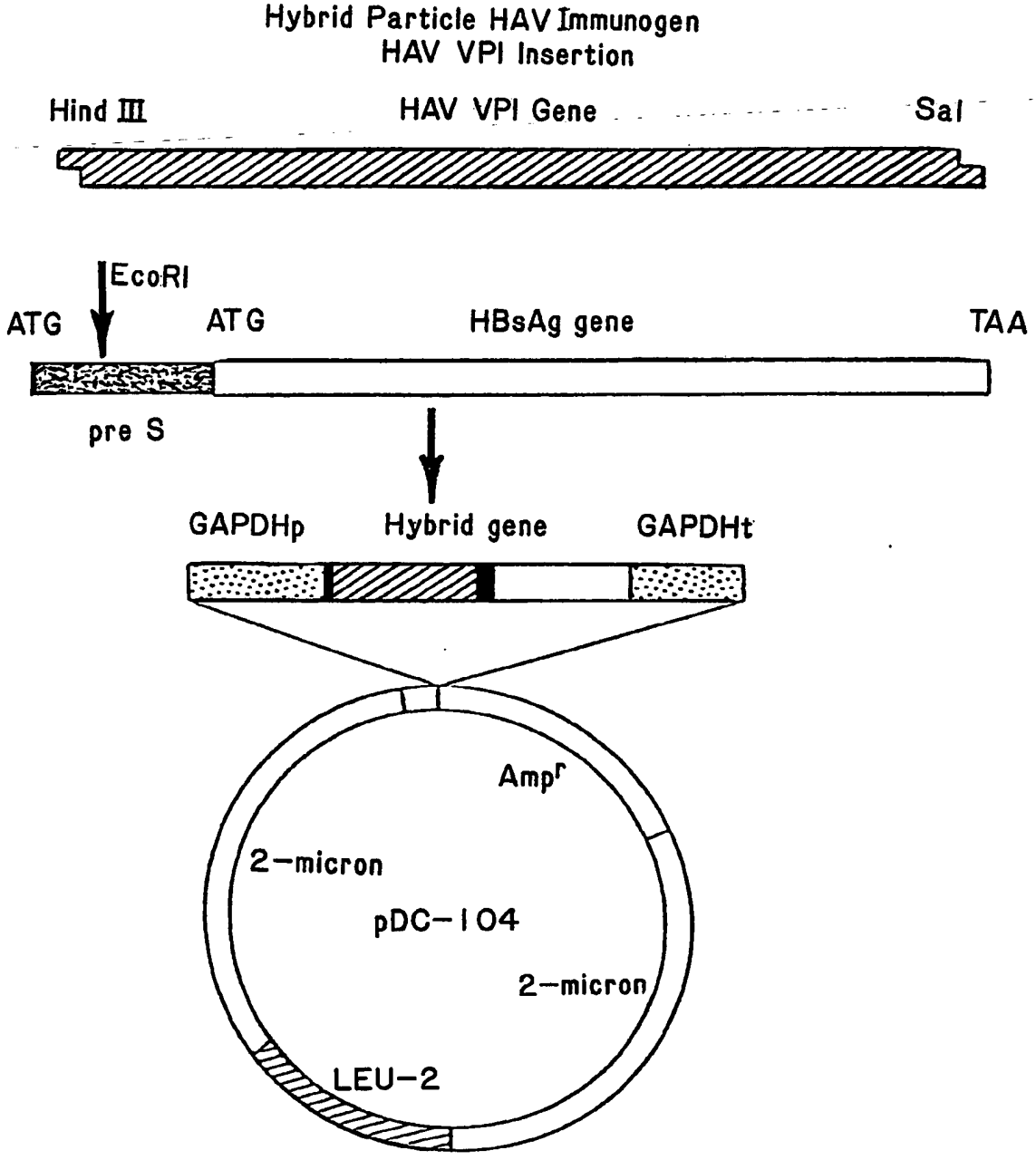
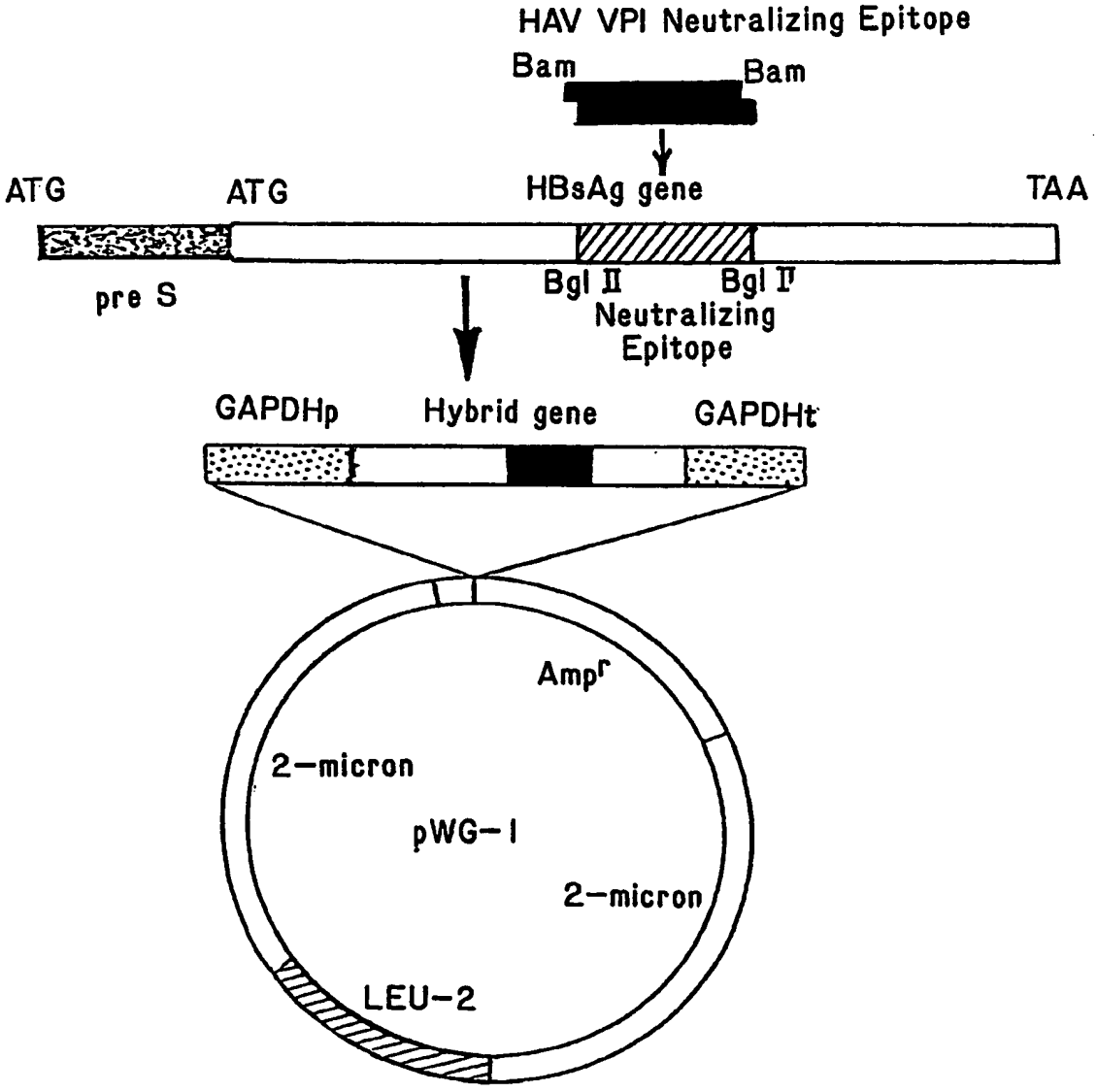

Hybrid Particle HAV Immunogen
HAV VPI Insertion
Hind III
HAV VPI Gene
Sal
EcoRI
ATG
ATG
HBsAg gene
TAA
pre S
GAPDHp
Hybrid gene
GAPDHt
Amp^r
2-micron
pDC-104
2-micron
LEU-2

FIG. 6

FIG. 7

Hybrid Particle HAV Immunogen
Neutralizing Epitope Substitution



WEST

P2A - 792-980

38(VPI/P2A) 779-798

39 792-811

40 799-818

41 810-829

42 823-842

(43) 834-853

44 922-941

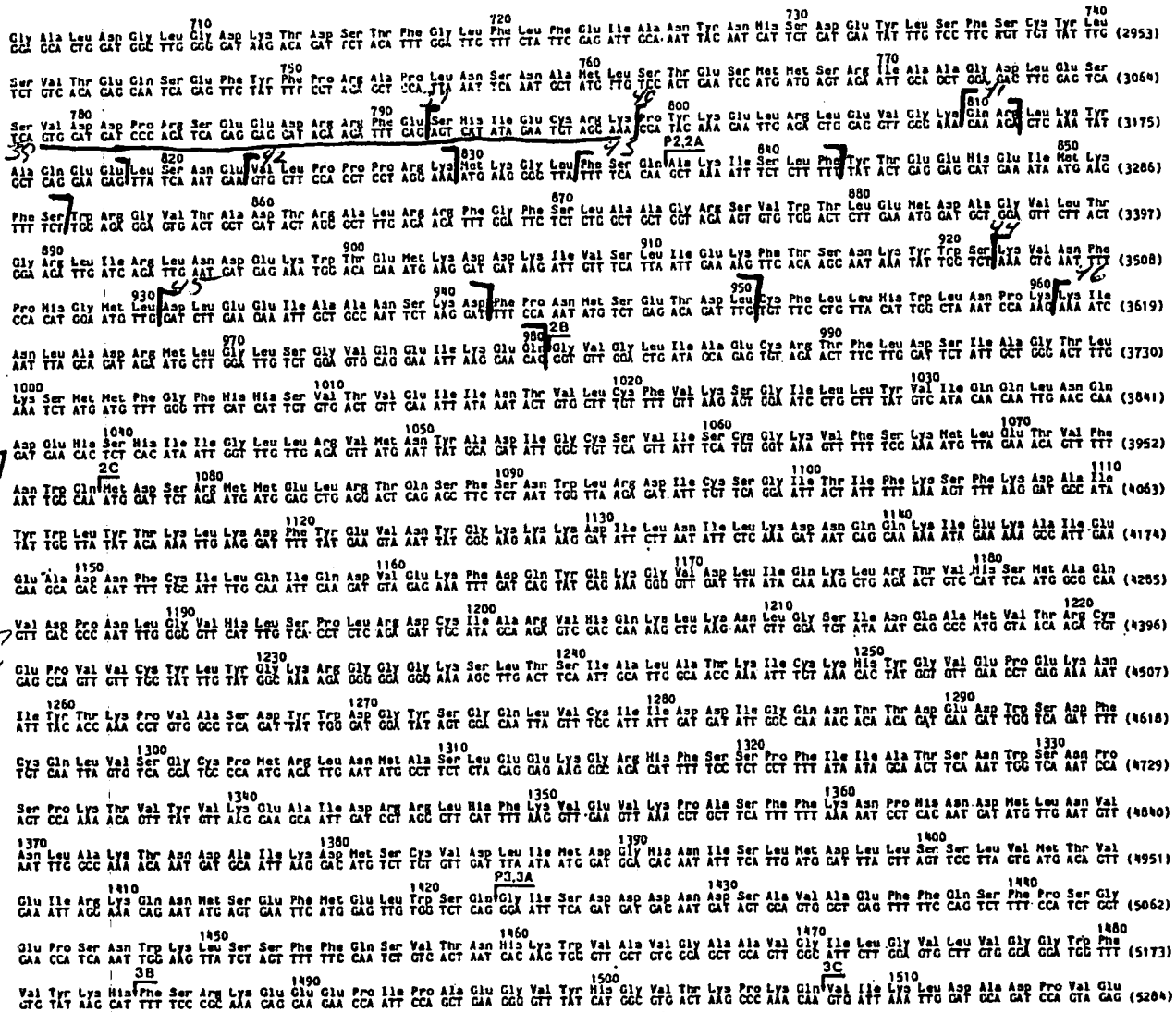
45 931-950

46 961-980

47 810-834

48 956-980

FIG. 1b



0 199 480